

FROM THE INSTITUTE OF ENVIRONMENTAL HEALTH,
UNIVERSITY OF LUND, SWEDEN

The Micronucleus as an Indicator of Genotoxic Exposure in Man

By

BENKT HÖGSTEDT

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THE MICRONUCLEUS AS AN INDICATOR
OF GENOTOXIC EXPOSURE IN MAN

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Abstract The micronucleus method was found to be a useful test for detection of genetic damage in humans exposed to environmental agents. Micronuclei were possible to study both in erythroblasts and polychromatic erythrocytes from the bone marrow as well as in cultured lymphocytes from the peripheral blood. When studying micronuclei in cultured lymphocytes preservation of the cytoplasm improved the precision, since it was easier to differentiate lymphocytes with micronuclei from other types of cells with segmentation or fragmentation of the nucleus, and to be sure that the micronucleus really belonged to the cell; also, a loss of micronuclei was avoided. The optimal culture time was between 80 and 88 h. The method error (variation coefficient) was 6-11% when 3,000 cells were analyzed. As an inter-observer variation existed, materials ought to be scored by the same observer. Micronuclei were found to be sensitive indices of factors such as age, smoking and of work environment agents such as petroleum products and low concentrations of ethylene oxide and styrene. Cells with abnormal karyotypes showed a higher tendency to induce micronuclei. Micronuclei in bone marrow cells and in lymphocytes showed associations with other methods indicating mutagenic impact in man. Correlation coefficients were, however, weak or lacking, which probably was due to a low precision and to differences in susceptibility of the parameters studied.		
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Date April 7, 1983

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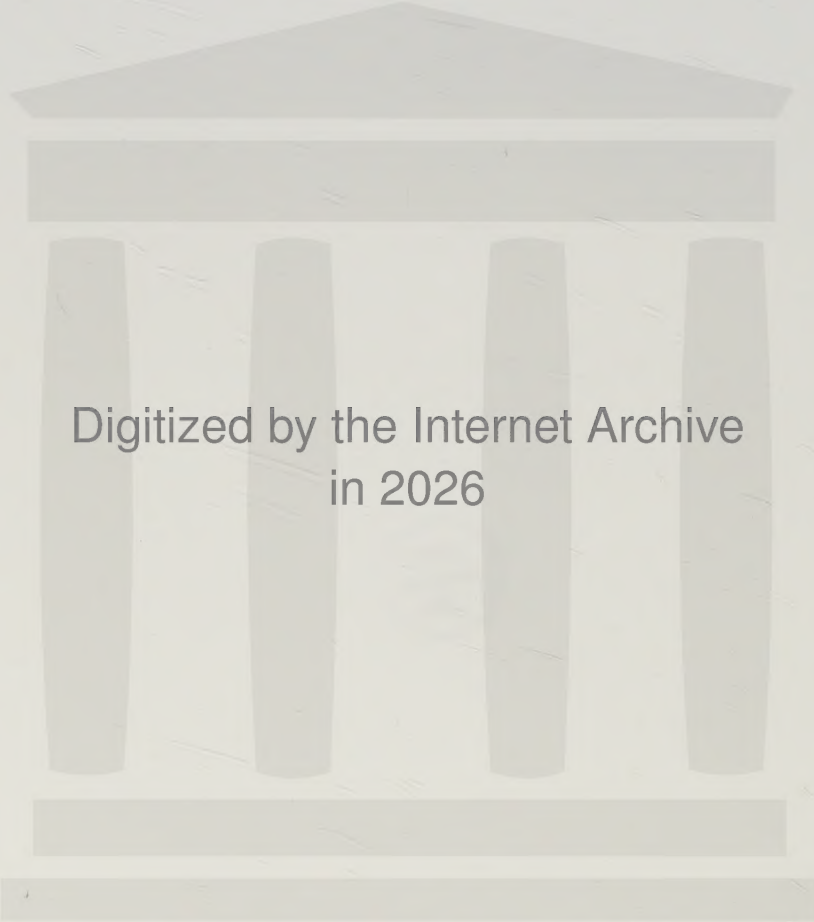
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This thesis is based on the following papers referred to by their Roman numerals:

I. Högstedt, B., Nilsson, P.G., and Mitelman, F. 1981. Micronuclei in erythropoietic bone marrow cells: Relation to cytogenetic pattern and prognosis in acute nonlymphocytic leukemia.- *Cancer Genetics and Cytogenetics* 3:185-193.

II. Högstedt, B. and Mitelman, F. 1981. The interrelations of micronuclei, chromosomal instability, and mutational activity in acute non-lymphocytic leukemia- A hypothesis.- *Hereditas* 95:165-167.

III. Högstedt, B., Gullberg, B., Mark-Vendel, E., Mitelman, F., and Skerfving, S. 1981. Micronuclei and chromosome aberrations in bone marrow cells and lymphocytes of humans exposed mainly to petroleum vapors.- *Hereditas* 94:179-187.

IV. Högstedt, B., Gullberg, B., Hedner, K., Kolnig, A-M., Mitelman, F., Skerfving, S., and Widegren, B. 1983. Chromosome aberrations and micronuclei in bone marrow cells and peripheral blood lymphocytes in humans exposed to ethylene oxide.- *Hereditas* 98:105-113.

V. Högstedt, B., Åkesson, B., Axell, K., Gullberg, B., Mitelman, F., Pero, R.W., Skerfving, S., and Welinder, H. 1983. Increased frequency of lymphocyte micronuclei in workers producing reinforced polyester resin with low exposure to styrene.- *Scandinavian Journal of Work, Environment & Health* 0:000-000.

VI. Högstedt, B. 1983. Micronuclei in lymphocytes with preserved cytoplasm - a method for assessment of cytogenetic damage in man.- Submitted for publication.

INTRODUCTION

It has been estimated that the total environmental impact, i.e. viruses, ionizing radiation and chemicals, causes a major part of all human cancers (Higginson and Muir 1977). Due to the rapid development of organic chemistry during recent decades, there has been an enormous increase of the number of chemical compounds. It has been estimated that the number of chemicals that are in common use is around 60,000 and is still increasing (Maugh 1978). This has lead to a fear of an increase of cancer in industrialized countries.

Recognition of a substance as a human carcinogen has earlier relied only on epidemiological evidence. The main drawback of epidemiological studies as a tool for monitoring cancer risks, is that the results come too late, mainly due to the long latency period of many tumors; the results may have historical interest only. Also, epidemiology is often a blunt instrument since the results may be confounded by various intervening factors.

In recent decades it has been realized that most influences with carcinogenic impact also have mutagenic effects. Thus, a close correlation has been found between chemical compounds with mutagenic activity in Ames' test and carcinogenic properties in experimental animals (e.g. McCann et al. 1975).

There is an apparently close correlation between mutagenic and clastogenic effects, i.e. different types of chromosomal aberrations. Also, Ishidate and Yoshikawa (1980) have concluded that mutagenicity tests based on clastogenic activity have high predictive value for the identification of carcinogens. Furthermore, it is known that individuals with the hereditary diseases

Fanconi's anemia, ataxia-telangiectasia and Bloom's syndrome, have a spontaneous high frequency of chromosome breakages and an elevated risk of cancer (Ray and German 1981). Also, the fact that most malignant tumors have chromosome abnormalities (Mitelman and Levan 1981), focuses the interest on the study of cytogenetic changes.

This gives an opportunity to study cytogenetic changes in humans exposed in vivo as a risk indicator of future cancer development. The practical possibilities now available are to study either numerical and structural chromosome aberrations or sister chromatid exchanges.

During the last decade the analysis of micronuclei in screening mutagenic substances in animals has been shown to be a very sensitive and much more rapid method than the cytogenetic methods mentioned (Schmid 1975). This thesis will concentrate on the possibility to use micronuclei as an indicator of genetic damage in man.

Micronuclei are well-known in haematological literature as Howell-Jolly bodies. Howell (1891) was the first to describe these bodies in peripheral erythrocytes of severely bled animals. Because of their staining properties Howell suggested that they were of nuclear origin. Some years later Jolly (1905) made the same observation.

The occurrence of Howell-Jolly bodies in the peripheral blood seems to be closely related to the function of the spleen, since this organ removes erythrocytes with such bodies from the circulation. It has been claimed that splenectomy also causes an increase of the formation of Howell-Jolly bodies in bone marrow cells (Hittmair 1969).

Howell-Jolly bodies are frequent in erythroblasts of patients with megaloblastic anemias, i.e. deficiency of vitamin B12 and folic acid

(Krogh Jensen 1977, Knuutila 1979). The biochemical background is thought to be a relative failure of thymidine synthesis, which interferes with the DNA metabolism.

Flidner et al. (1964) were the first to use micronuclei as an indicator of chromosome damage. They studied a group of individuals who accidentally had been gamma-irradiated. In repeated bone marrow aspirations they analyzed the frequencies of micronuclei in erythroblasts and were able to give an exact picture of the rise and fall of micronuclei after a single chromosome damaging event (fig. 1).

Heddle (1973) and Schmid (1975) presented "the micronucleus test" as a method for testing chemicals with mutagenic effects in animals. Schmid showed that the polychromatic erythrocytes in the bone marrow were suitable for analyzing micronuclei since the main nucleus of the cells was lost. The polychromatic cells could be differentiated from older erythrocytes as they stained bluish in May-Grunwald-Giemsa stain. They have, compared to other bone marrow cells, a well defined life span. The micronucleus test has proved to be a sensitive and rapid method and has also showed a strong association with the results of bacteriological testing (Jenssen and Ramel 1980).

Further human studies of micronuclei in bone marrow cells have been made by Goetz et al. (1975, 1976), Krogh Jensen and Huttel (1976) and by Krogh Jensen and Nyfors (1979), who have investigated the effects of cytostatic drugs in groups of patients. Since polychromatic erythrocytes in humans stain weaker than in laboratory animals, Krogh Jensen and coworkers has found them difficult to use and therefore analyzed micronuclei only in erythroblasts. However, the possibility to use the method for studying chemical environmental exposures has not been systematically

evaluated.

It has been observed that renal cancer and seminoma cells may form micronuclei in vivo (Söderström 1966). Malignant tumors may have abnormal karyotypic patterns and cells with an abnormal karyotype (e.g. Down's syndrome) are more prone to develop cytogenetic damage (for references see paper I and II). Therefore, it was interesting to investigate, if there is any relation between an abnormal karyotypic pattern and the frequencies of micronuclei in malignant cells.

The possibility to study micronuclei in cultured lymphocytes from the peripheral blood has been investigated by Countryman and Heddle (1976). They showed that in vitro exposure to ionizing radiation gives a dose related increase of micronuclei in cultured lymphocytes which was most pronounced in cells with an abnormal karyotypic pattern. This was also found with mitomycin C (Heddle et al. 1978a). Norman et al. (1978) found an increased level of micronuclei in patients after X-ray examinations with iodine-containing contrast media. Linnainmaa et al. (1978) obtained a dose-related effect on micronuclei by in vitro exposure to styrene. These authors all scored micronuclei in cell preparations where the cytoplasm had been destroyed by hypotonic treatment.

This method, which means that the lymphocytes are treated with hypotonic saline, is certainly useful in experimental situations where it is possible to induce high levels of micronuclei but probably less sensitive in in vivo exposed humans with low levels of micronuclei. Thus, it is difficult to differentiate lymphocytes with micronuclei from cells with segmentation and fragmentation of the nucleus. Furthermore some micronuclei are probably torn away from the neighborhood of the original nucleus by the hypotonic treatment and nuclear fragments from other cells are easily taken for micronuclei.

The induction of micronuclei is a general phenomenon that may occur in all types of cells. As mentioned, bone marrow cells and lymphocytes have been studied in man. Fibroblasts (Midander 1982) and buccal mucosa cells (Stich and Rosin 1983) have also been used.

A micronucleus is formed during cell division from chromosome material which has lost its contact with the spindle apparatus. Such material is either acentric fragments or whole chromosomes. Micronuclei therefore reflect damage either to the chromosomes (breakage events) or to the spindle apparatus (lagging of chromosomes). For review see the report of Jenssen (1982). It has also been shown, as could be expected, that acentric fragments give smaller micronuclei than whole chromosomes (Yamamoto and Kikuchi 1980).

After an acute damage to the bone marrow or to any other dividing cell line micronuclei will be detected in interphase cells after the first division. In the bone marrow this increase is detected in the first 24 h (fig. 1). The relative frequency of micronuclei is the highest after the first cell division and will probably be more rare in further generations. There is a progressive dilution of the damaged cell population with normal cells as the culture proliferates. These circumstances cause a rise and fall of micronuclei frequencies, which are important to investigate when the culture time of lymphocytes is discussed. It could be mentioned that there have been different opinions on the optimal culture time (Heddle et al. 1978b).

% micronuclei

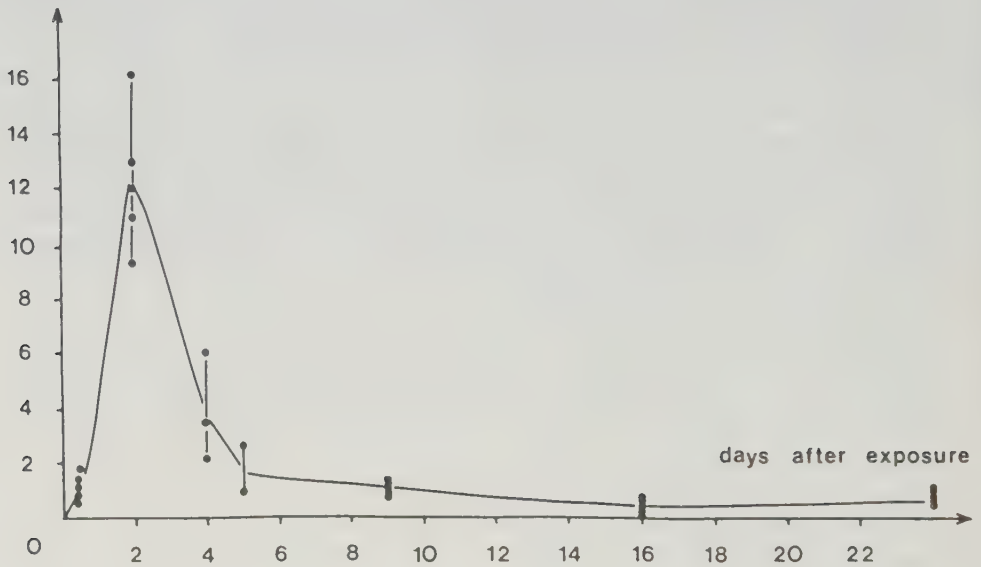


Fig. 1.

Frequencies of micronuclei in erythroblasts in five individuals accidentally exposed to 3 Gy of gamma-irradiation (Flidner et al.1964). Repeated bone marrow aspirations were made at times indicated. Published by permission of professor T.M. Flidner.

AIMS OF THE THESIS

1. To investigate whether micronuclei in erythropoietic bone marrow cells and peripheral blood lymphocytes are useful tools for detection of mutagenic effects in man of environmental agents.
2. To modify the method for studying micronuclei in peripheral blood lymphocytes in order to obtain a better precision of the cytological analysis; to study the method error and the problem of the optimal culture time.
3. To study the relation between the formation of micronuclei and the presence of an abnormal karyotypic pattern.
4. To compare the results of micronuclei in erythropoietic bone marrow cells and lymphocytes with other parameters indicating mutagenic impact (chromosomal aberrations, sister chromatid exchanges and unscheduled DNA synthesis).
5. To investigate how age, smoking and work environment exposures (petroleum vapors and low levels of ethylene oxide and styrene) influence the induction of micronuclei in bone marrow cells and lymphocytes in humans.

INDIVIDUALS STUDIED

Patients with acute non-lymphocytic leukemia (ANLL)

Twenty-five patients (11 women and 14 men), whose ages ranged from 15 to 84 a (median age 57 a), were investigated at the time of diagnosis, i.e. none of the patients had received any anti-leukemic treatment. Karyotypes of bone marrow cells were determined by means of the trypsin-Giemsa banding technique.

According to the cytogenetic pattern of the leukemic cells the patients were divided into three groups:

1. Patients with abnormal bone marrow metaphases only (AA patients)
2. Patients with both normal and abnormal bone marrow metaphases (AN patients)
3. Patients with normal bone marrow metaphases only (NN patients).

Workers exposed to petroleum vapors and controls

This group of workers was chosen as there were several epidemiological studies showing an increased risk of cancer caused by occupational exposures to petroleum products and organic solvents (Brandt et al. 1978, Olsson and Brandt 1979, Thomas et al. 1980, Flodin et al. 1981).

Exposed workers. Sixteen men (aged 27-58, median 35 a) working as tank cleaners were studied. Ten were smokers, 5 were ex-smokers, only one had never smoked. The work environment is presented in detail in III.

Controls. Ten bus drivers (8 men and 2 women) whose ages ranged from 25 to 52 (median 37) a, were used as controls. Five were smokers.

Workers_exposed_to_ethylene_oxide

Ethylene oxide is a well-known mutagen and has proved to be a carcinogen in experimental animals (for references see paper IV). Studies by Hogstedt et al. (1979a,b) indicated that ethylene oxide also may cause malignancies in man. Only a few cytogenetic studies on workers exposed to ethylene oxide have been published. Garry et al. (1979) and Lambert et al. (1982) have found increased levels of SCE and Thiess et al. (1981) showed higher levels of chromosome aberrations in lymphocytes. In these investigations the exposure levels were higher than in our study.

Exposed_workers_in_factory_I. Eighteen individuals (17 women and 1 man) aged 19 to 55 (mean 37) a, who had been exposed to ethylene oxide for 0.5-8 (mean 3.2) a, producing medical equipment. Four were sterilizing and 12 packing personnel. Nine were smokers.

Controls_A (micronuclei in lymphocytes). Eleven women aged 21-58 (mean 44) a, who worked in another department of the same factory. Six were smokers.

Controls_B (micronuclei in bone marrow cells). See "workers exposed to petroleum vapors -controls".

Exposed_workers_in_factory_II. Ten men aged 20-55 (mean 36) a, who had been exposed for 0.5 to 8 (mean 1.7) a, producing medical equipment. Seven of them were truck drivers and/or packing personnel and the rest were sterilizing personnel. Five were smokers.

Controls. Nine male workers from another department of the same factory were used as controls. Their ages ranged from 20 to 44 (mean 29) a. Four were smokers.

Workers exposed to styrene

Styrene and its metabolite styrene-7,8-oxide are known mutagens and have also been shown to be carcinogens in animals (for references see paper V). However, convincing evidence of human carcinogenicity is still lacking.

Exposed workers. Thirty-eight men (19-63, median 37.5 a), who had been exposed to styrene in a factory producing fiberglass reinforced unsaturated polyester resin for 1-23 (median 6) a. Seventeen were smokers.

Controls. Twenty male workers (22-59, median 34 a) employed in a mechanical industry. Eight were smokers.

STATISTICAL METHODS

The Mann-Whitney U test and the Kruskal-Wallis test of ordered alternatives were used in papers I and III to test whether different groups had been drawn from the same population. The Spearman rank correlation test was used for the analysis of correlation. These tests are of the nonparametric type and were preferred since they do not require normal distribution of the variables (Siegel 1956, Bradley 1968).

To separate the effects of a multifactorial influence and to eliminate bias, multivariate analysis (analysis of variance, covariance and multiple regression) was used (Lindman 1974, Edwards 1979). This analysis included factors like age, smoking, and occupational chemical exposure. The demand of normally distributed variables in this analysis was met by logarithmic transformations of the original values.

All tests were two-sided. Statistical significance denotes $p < 0.05$.

METHODOLOGICAL ASPECTS ON THE STUDY OF MICRO-NUCLEI

Micronuclei in erythropoietic bone marrow cells

A. Preparation

Bone marrow cells were obtained by sternal puncture. The cells were suspended in a mixture of equal parts of isotonic phosphate buffer (pH 7.0) and fetal calf serum. After being shaken, the cells were pelleted and the supernatant was removed. The pellet was resuspended in a few drops of buffer and calf serum solution. The cells were smeared on slides, air dried and stained with May-Grunwald-Giemsa stain. The bone marrow aspirates from the leukemic patients were smeared directly on slides without any dilution. In every preparation 1,000 interphase cells of both types (see below) were analyzed.

B. Analysis of erythroblasts

Most of the cells scored were of the polychromatic type, rather few of more immature forms. The erythroblasts were always easy to differentiate from other cells in the bone marrow. In man this cell type has enough cytoplasm to harbour micronuclei quite separated from the main nucleus. Generally, there were fewer micronuclei in erythroblasts than in polychromatic erythrocytes, the exception being the leukemic patients.

The size of micronuclei varied somewhat with the size of the main nucleus. Compared to micronuclei in polychromatic erythrocytes they were generally larger. The structure and color of the micronuclei were close to the main nucleus.

In normal unexposed humans, Krogh Jensen and Nyfors (1979) and Knuutila (1979), both have found a range of 0-4 o/oo micronuclei in erythroblasts. The range of our control group is 0-6 o/oo (papers III and IV).

C. Analysis of polychromatic erythrocytes

A micronucleus in a polychromatic erythrocyte is a rounded, sharply outlined body around 1 μ m across. It stained deep blue or almost black.

The polychromatic erythrocyte, which has no nucleus, has special staining properties in May-Grunwald-Giemsa during approximately 24 h and stains light blue or grey. The staining procedure is of great importance to get a proper differentiation of these cells. However, the identification of the polychromatic erythrocytes in humans is more difficult than in many experimental animals.

Thus, Krogh Jensen and Huttel (1976), Krogh Jensen and Nyfors (1979) and Knuutila (1979) have found these cells impossible to use while Goetz et al. (1975,1976) have only used polychromatic erythrocytes in their investigations. In the present investigations both erythroblasts and polychromatic erythrocytes were analyzed.

As can be seen in fig. 2, there is a very good correlation between the two analyses of the same preparations ($r = 0.93$, $N = 11$). The preparations were recoded between the two scores made by the same observer.

There were also significant correlations between the frequencies of micronuclei in erythroblasts and in polychromatic erythrocytes in all the different materials (papers I,III,IV; $r = 0.41$, $r = 0.64$, $r = 0.62$, respectively).

2. score

Micronuclei in polychromatic
erythrocytes (‰)

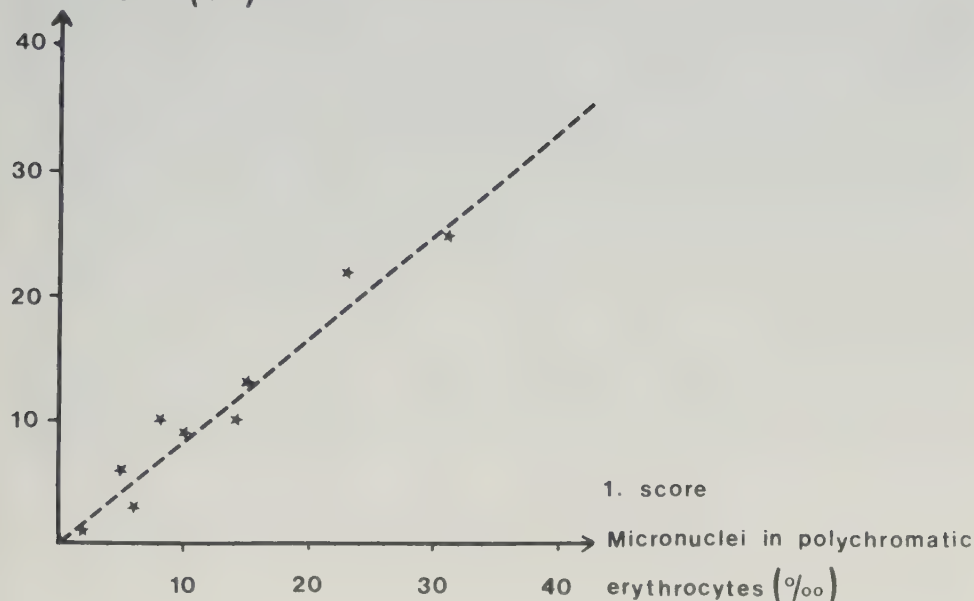


Fig. 2.

Frequencies of micronuclei in polychromatic erythrocytes in 11 individuals. Two scores were made blindly by the same observer. The Spearman rank correlation coefficient was 0.93.

Therefore, in addition to micronuclei in erythroblasts, it is also quite possible in humans to use the information on micronuclei in polychromatic erythrocytes.

In literature, there are no records of micronuclei in polychromatic erythrocytes in normal individuals. Goetz et al. (1975, 1976) have found 3.1 and 3.9 o/oo as mean values of two groups of patients with various types of malignant diseases before treatment, which should be compared to the mean of our controls, 3.2 o/oo (see papers III and IV).

Method error

The relative method error (variation coefficient = $100 \times \text{standard deviation} / \text{mean}$) of scoring micronuclei in polychromatic erythrocytes, calculated on the above mentioned 11 paired observations (fig. 2) was 17.7%. This can be regarded as the total method error since the observations are made on preparations of cells representing in vivo conditions.

Micronuclei in peripheral blood lymphocytes

A. In cells with preserved cytoplasm

Heparinized whole blood or leukocytes from the "buffy coat" layer was added to McCoy's 5A medium with 20% new born calf serum. After incubation in 37 °C the cells were centrifuged. The medium was removed and the pellet was suspended in an equal volume of medium, smeared on slides, air dried, and stained in May-Grunwald-Giemsa.

The slides were examined with an oil immersion lens with a total magnification of $\times 1,000$. The

lymphocyte nucleus stained red-brown and the cytoplasm blue. The micronuclei were of different sizes but had to be not larger than one third of the main nucleus and had to be clearly separated from it. Lymphocytes with micronuclei could be differentiated from other nucleated cells with fragmentation or segmentation of the nucleus.

This method was used in papers V and VI.

Pertinent results and comments on methodological aspects (cytological findings, culture time, relation between whole blood vs "buffy coat" cultures, method error, inter-observer variation) are given in paper VI. The optimal culture time was found to be between 80 and 88 h. The method error (variation coefficient) was 6-11% when 3,000 cells were analyzed. There was a close correlation between the judgements of two observers, though a systematic difference existed.

B. In hypotonically treated cells

The cells were cultured for 72 or 96 h according to the microculture technique used in our laboratory (see papers III, IV) and then treated for 1 h with colchicin, and thereafter with 0.075 mol/l KCl, and finally fixed in methanol:acetic acid (3:1). The cells were spread on slides, air dried and stained in Giemsa. The frequency of micronuclei was estimated by scoring 2,000 lymphocyte nuclei. The preparations were examined with the magnification of $\times 1,000$. Almost the same criteria as above were used for classification of micronuclei. However, since the cytoplasm was lacking, the micronuclei had to be situated within two nuclear diameters from the main nucleus.

Colchicin treatment of the cultures was made as the preparations were also used for scoring structural and numerical chromosome aberrations.

This treatment did not affect the formation of micronuclei as new interphase cells were not formed after colchicin had been added.

This method was used in papers IV, V and VI.

Comment

As can be seen in paper VI, fig. 1 an increased frequency of micronuclei in cultured lymphocytes can be detected after 48 h. The maximum appears after 72-96 h and then the frequency decreases. This is comparable to the findings on bone marrow micronuclei (p. 14, fig. 1), though the time sequence is different. As mentioned in "Introduction" the relative frequency of micronuclei is highest in interphase cells after the first division. The decreased frequency is caused by a dilution of the micronucleated cell population with normal lymphocytes.

FACTORS INFLUENCING THE VARIATION OF MICRO-NUCLEI

Age

The effect of age has been studied by multivariate analyses in individuals exposed to ethylene oxide, and styrene and in controls (IV, V, and VI).

In the group of ethylene exposed individuals and in controls there was no statistically significant effect of age on the micronuclei parameters. Chromosome breaks in lymphocytes and also total number of aberrations in lymphocytes displayed almost significant age effects ($p = 0.055$ and $p = 0.064$, respectively; not earlier published).

Styrene exposed individuals and their controls showed age effects in all three measurements of micronuclei (micronuclei in whole lymphocytes cultured for 72 h, $p = 0.048$; micronuclei in whole lymphocytes cultured for 96 h, $p = 0.11$; micronuclei in hypotonically treated lymphocytes, $p = 0.002$).

According to table 1 the percentage of the total variance explained by age ranged from 0 to 20% in the micronuclei parameters of the present study. Age seems to have some effect in almost all parameters.

There are some earlier cytogenetic studies in which an age effect has been found (for review see Hedner et al. 1983a), but in most studies, age has not been considered in the statistical analysis.

Smoking

In the three investigations where occupational exposure was considered (papers III, IV, V), smokers generally had higher mean levels of damage in all cytogenetic parameters, though the statistical significance differed.

In the group of workers exposed to petroleum vapors (paper III) smokers had higher levels of damage in almost all cytogenetic parameters studied.

In the ethylene oxide exposed group and controls (paper IV), a multivariate analysis revealed a statistically significant effect of smoking on micronuclei in erythroblasts and in hypotonically treated lymphocytes.

In the styrene exposed group and controls, there was a statistically significant effect of smoking in micronuclei in lymphocytes with preserved cytoplasm cultured for 96 h.

As can be seen in table 1 the percentage of the total variance explained by smoking, ranges from 0 to 17% in different variables of micronuclei. Many cytogenetic studies, particularly regarding sister chromatid exchanges, have shown a significant influence of smoking, though some have not (Lambert et al. 1978, Husgafvel-Pursiainen 1980, Hedner et al. 1983b). The somewhat different results in this respect may be explained by the combination of limited influence of smoking and a considerable method error that interferes with the possibility to obtain statistical significance.

Table 1
Variance (%) explained by age, smoking and occupational exposure.

	Ethylene oxide exposed individuals and controls			Styrene exposed individuals and controls		
	Micronuclei in			Micronuclei in		
	erythro- blasts (N=27)	polychro- matic erythrocytes (N=27)	hypotonic- treated lymphocytes (N=48)	whole lymphocytes 72 h (N=58)	whole lymphocytes 96 h (N=58)	hypotonic- treated lymphocytes (N=58)
Age	2	2	0	7	4	20
Smoking	16	1	17	2	10	0
Occupational exposure	13	19	0	3	10	1
Total	31	22	17	12	24	21

Work_environment_factors

Workers exposed to petroleum vapors

The exposed workers showed a statistically significant increase of micronuclei in erythroblasts and polychromatic erythrocytes compared to the controls (paper III, fig. 2). They were exposed to many chemical compounds with known or suspected mutagenic activity, i.e. petrol and petrol additives, light and heavy oils, organic solvents and heavy metals. Occupational history and a few measurements of air levels suggested that the tank cleaners as a group suffered heavy chemical exposure.

Individuals exposed to ethylene oxide

In the group of ethylene oxide exposed workers in factory I there were significantly increased frequencies of micronuclei in erythroblasts and polychromatic erythrocytes compared to controls (paper IV, fig. 2). Micronuclei in lymphocytes without cytoplasm, however, did not show any significant effect of exposure in workers from factory I and II.

The exposure levels did not exceed 1 ppm TWA (time weighted average) at the time of investigation. These levels were far below the current Swedish TLV (threshold limit value) for ethylene oxide (5 ppm). In the two plants investigated, ethylene oxide was mixed with methyl formiate and a fluorocarbon compound (Freon 12), respectively. Neither of the compounds, however, have any known mutagenic effects.

Individuals exposed to styrene

According to the multivariate analysis there was a statistically significant effect of styrene in the exposed group compared to the controls on micronuclei in lymphocytes with preserved cytoplasm cultured for 96 h (paper V, fig. 1).

In the exposed group no one had been exposed to TWA levels exceeding 40 ppm during the last 5 a before the investigation. When this took place the individual TWA levels ranged from 1-36 ppm with a mean of 13 ppm. The air levels of styrene correlated well to low levels of mandelic acid in the urine (range 9-316, mean 65 mg/g creatinine).

Comment

It is important to stress that even when the factors studied (age, smoking and work environment) are considered, the total explained variance was only 12-31% in the different parameters (table 1). This indicates that other factors may be operating.

Abnormal karyotype

The results in papers I and II suggested that an acquired abnormal karyotype may be associated with an increased sensitivity to mutagenic influences, which is in accordance with earlier findings in cells with constitutional karyotypic abnormalities (for references see paper I and II).

As reported in paper VI there was a relation between micronuclei in lymphocytes and cells with more than 46 chromosomes. The explanation of this association might be that a lagging chromosome either forms a micronucleus or is included in one

of the daughter cell nuclei as an extra chromosome. In addition, the cells with aneuploidy probably are more easily damaged and induce micronuclei at a higher rate compared to normal ones.

According to paper I (fig. 1) there were obvious differences between AA and NN patients in the frequencies of micronuclei in erythropoietic bone marrow cells. Also, in the AN group a correlation between the percentages of pathological metaphases and the frequencies of micronuclei was discernable.

It was further found that the frequency of micronuclei was negatively associated with the survival time of the leukemic patients. We suggested that one possible explanation of the poor survival of patients with abnormal chromosome pattern/high frequency of micronuclei could be the increased mutagenic sensitivity in these cells imposing a higher risk of further mutations and an accelerated malignant progression (papers I and II).

It has recently been demonstrated that certain karyotypic abnormalities may only be present in myeloid cells but not in erythropoietic ones (Berger et al. 1982). The micronuclei were studied in the erythropoietic cell line. As there was an association between micronuclei and abnormal karyotype it is likely that the abnormality in the cases with high levels of micronuclei was present in the erythropoietic cells. Therefore, the worse prognosis of patients with high frequencies of micronuclei also could be caused by the erythropoiesis being affected by the disease.

The mean frequency of micronuclei in patients of group NN were somewhat higher compared to our controls (in erythroblasts 3.3 ± 1.0 o/oo, in polychromatic erythrocytes 4.1 ± 3.2 o/oo,

respectively; not earlier published) but the differences are only statistically significant as regards micronuclei in erythroblasts ($0.002 < p < 0.02$; Mann-Whitney U Test). It could also be mentioned that Goetz et al. (1975, 1976) have found micronuclei frequencies of 3.1 and 3.9 o/oo in polychromatic erythrocytes as mean values of two groups of patients with various types of malignant diseases before treatment, which is rather close to the present findings in leukemic NN patients and controls. Therefore, it is not possible to answer the question whether the leukemia per se gives an increased frequency of micronuclei in the bone marrow cells.

ASSOCIATION BETWEEN MICRONUCLEI AND OTHER PARAMETERS INDICATING MUTAGENIC EFFECTS

In workers exposed to petroleum vapors and controls

Both micronuclei in erythropoietic bone marrow cells and chromosome aberrations in lymphocytes showed essentially the same results, i.e. exposure associated differences between the groups of tank cleaners and controls (paper III).

There were no statistically significant correlation coefficients between the cytogenetic parameters in this study.

In workers exposed to ethylene oxide and controls

The exposed workers had significantly increased frequencies of micronuclei in erythroblasts and polychromatic erythrocytes and in chromosome aberrations in lymphocytes as well. All parameters showed higher mean frequencies for smokers than for non-smokers but the smoking effect was statistically significant only in micronuclei in erythroblasts and in hypotonically treated lymphocytes (paper IV).

The increased levels of cytogenetic damage in this study were paralleled by a decreased carcinogen induced unscheduled DNA synthesis, probably due to an inhibition of repairing enzymes caused by the alkylating effects of ethylene oxide (Pero et al. 1981, Pero et al. 1982a).

Further, positive and statistically significant correlation coefficients existed between micronuclei in erythroblasts and chromosomal breaks in lymphocytes and between micronuclei in polychromatic erythrocytes and the number of total chromosome aberrations in lymphocytes; the micro-

nuclei in hypotonically treated lymphocytes did not show any correlations with the cytogenetic variables studied (paper IV, table 4).

In styrene exposed workers and controls

Age was associated with significant effects in micronuclei in lymphocytes without cytoplasm and in lymphocytes with preserved cytoplasm cultured for 72 h (paper VI). Micronuclei in lymphocytes with preserved cytoplasm cultured for 96 h displayed a pronounced effect of styrene exposure (paper V). This was also the case with carcinogen induced unscheduled DNA synthesis ($p = 0.001$; Pero et al. 1982b).

There were slight but significant correlations between micronuclei in lymphocytes with preserved cytoplasm cultured for 72 and 96 h ($r = 0.30$, $p = 0.024$) and between micronuclei in lymphocytes with preserved cytoplasm cultured for 72 h and in cells treated with hypotonic saline ($r = 0.26$, $p = 0.044$). These two parameters correlated significantly with cells with more than 46 chromosomes ($r = 0.27$, $p = 0.044$; $r = 0.30$, $p = 0.024$; respectively). The carcinogen induced values of unscheduled DNA synthesis did not correlate significantly to any of the micronuclei parameters.

Comments

The method error is probably prominent in all cytogenetic measurements. Thus, it was not surprising that there were no consistent correlations between the different parameters. Furthermore, to obtain a correlation it is needed that both parameters are affected by the exposure. In addition, if an exposure effect has a different time sequence in the parameters, there will be no correlation.

GENERAL CONCLUSIONS

1. The micronucleus method is a useful test for detection of genetic damage in humans exposed to environmental agents. Micronuclei may be studied both in erythroblasts and polychromatic erythrocytes from the bone marrow as well as in cultured lymphocytes from the peripheral blood.
2. When studying micronuclei in cultured lymphocytes preservation of the cytoplasm improves the precision, since it is easier to differentiate lymphocytes with micronuclei from other types of cells with segmentation and fragmentation of the nucleus, and to be sure that the micronucleus really belongs to the cell; also, a loss of micronuclei is avoided. The optimal culture time is between 80 and 88 h. The method error (variation coefficient) is 6-11% when 3,000 cells are analyzed. As an inter-observer variation exists, materials ought to be scored by the same observer.
3. Micronuclei are associated with age smoking and of work environment agents (petroleum vapors, low concentrations of ethylene oxide and styrene).
4. Cells with abnormal karyotypes show a higher tendency to induce micronuclei.
5. Micronuclei in bone marrow cells and in lymphocytes showed associations with other methods indicating mutagenic impact in man. Correlation coefficients are, however, often weak or lacking.

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Micronuclei in Erythropoietic Bone Marrow Cells: Relation to Cytogenetic Pattern and Prognosis in Acute Nonlymphocytic Leukemia

Benkt Högstedt, Per Gunnar Nilsson, and Felix Mitelman

ABSTRACT: The bone marrow karyotype and the frequency of micronuclei in erythropoietic bone marrow cells (Howell–Jolly bodies) were determined in 25 adults with acute nonlymphocytic leukemia (ANLL). Ten patients had exclusively normal diploid bone marrow cells; 11 had a mixture of normal and abnormal cells, and 4 had abnormal bone marrow metaphases only. The frequency of micronuclei ranged from 3 to 28/2000 erythropoietic bone marrow cells (median 10). The number of micronuclei was significantly higher in patients with abnormal metaphases than in those with normal metaphases; in patients with a mixture of normal and abnormal bone marrow metaphases there was an association between the frequency of abnormal metaphases and the number of micronuclei. A striking difference in median survival time was found between patients with low and high numbers of micronuclei, irrespective of the cytogenetic bone marrow patterns. Patients with fewer than 10 micronuclei per 2000 erythropoietic bone marrow cells had a median survival of 148 days; those with more than 10/2000 had a median survival of only 34 days ($0.002 < p < 0.02$).

INTRODUCTION

About 50% of patients with acute nonlymphocytic leukemia (ANLL) have chromosomal abnormalities in their bone marrow cells; the other 50% have no detectable chromosomal changes [1]. The presence of chromosomal abnormality has prognostic implications: patients with only normal bone marrow cells have a better prognosis than patients with abnormal cells only or a mixture of normal and abnormal cells [2–5].

The frequency of micronuclei in erythropoietic bone marrow cells provides an opportunity to study indirectly the chromosomal stability of a defined bone marrow cell population throughout the whole cell cycle, i.e., not only in dividing cells. Acentric fragments or other chromosomal material excluded from anaphase groups and the resulting daughter nuclei may form separate micronuclei that can readily be observed in the cytoplasm of erythropoietic bone marrow cells. Scoring micronuclei in erythropoietic bone marrow cells is relatively simple, and large numbers of cells can

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be analyzed. In our study of patients with ANLL we found a correlation between the frequency of micronuclei and the bone marrow karyotype, on one hand, and survival time on the other.

MATERIALS AND METHODS

The 25 patients in this study were treated at the Department of Internal Medicine, University Hospital, Lund, Sweden, from 1973 to 1978. The group comprised 11 female and 14 male patients whose ages ranged from 15 to 84 years (median age 57 years). All patients were investigated at the time of diagnosis, i.e., none of the patients had received any antileukemic treatment. All cases were classified cytologically according to the criteria established by the French-American-British (FAB) cooperative group [6]. No patient had megaloblastic bone marrow.

The chromosomes were studied in direct bone marrow preparations by means of the trypsin-Giemsa banding technique as described by Mitelman et al. [7]. Karyotypes were determined by photographic analysis in as many cells as possible. An average of 15 metaphases from each patient were karyotyped in detail (no fewer than 5 metaphases were karyotyped from any one patient). An abnormal clone was defined as two cells with the same extra chromosome or structural rearrangement or three cells with the same missing chromosome. The main clonal karyotypic abnormalities are given in Table 1; the detailed karyotypic findings in all patients except Cases No. 1, 11, 24, and 25 were reported previously [5].

The patients were divided into three cytogenetic groups:

1. Patients with abnormal bone marrow metaphases only (AA-patients) (Cases No. 1-4);
2. Patients with both normal and abnormal bone marrow metaphases (AN patients) (Cases No. 5-15);
3. Patients with normal bone marrow metaphases only (NN patients) (Cases No. 16-25).

Micronuclei were counted in bone marrow smears stained according to the May-Grünwald-Giemsa method. The slides used were routine preparations of different quality and of different age, some more than 5 years old. In many cases only one slide was available for analysis. In each case 1000 erythroblasts, primarily of the polychromatic type, and 1000 polychromatic erythrocytes were analyzed. In three patients, Cases No. 12, 19, and 23, it was only possible to count 300, 1000, and 800 cells, respectively. In these patients the staining quality was good but the total number of cells was limited. Since fewer than 2000 cells were recorded in these three cases, the results presented in Table 1 and Figures 1-3 have been adjusted to equal the occurrence in 1000 erythroblasts and 1000 polychromatic erythrocytes, respectively. The bone marrow smears were examined without any knowledge of clinical or chromosomal data.

A micronucleus in a polychromatic erythrocyte was defined as a rounded, bluish or almost black structure with a sharp contour, about $1\ \mu$ in diameter. The micronucleus in an erythroblast is somewhat larger and has the same color as the main nucleus. Only interphase cells were analyzed when the number of micronuclei was scored.

RESULTS

The sex and age of the patients, the cytologic leukemia type (FAB), the type of chromosomal abnormality and the percentage of abnormal metaphases, the number of micronuclei in erythroblasts and polychromatic erythrocytes, and the survival time calculated from the day of diagnosis to the day of death are presented in Table 1. Fig-

Table 1 Clinical, cytogenetical, and cytological data in 25 patients with acute nonlymphocytic leukemia

Patient No.	Age (yr)	Sex	Cytologic type (FAB)	Bone marrow karyotype		No. of micronuclei in			Survival (days)
				Abnormal clone	%	Erythroblasts	Polychromatic erythrocytes	Total	
1	84	M	AML	44,XY,-16,-20,4q-,5q-,17q-	100	20	8	28	18
2	15	F	AML	46,XX,t(8;16)	100	9	17	26	5
3	57	M	AML	44,XY,t(5;12),i(18q),-19	100	9	13	22	27
4	77	F	AMMoL	46,XX,i(17q)	100	2	12	14	365
5	32	M	AML	45,XY,+2,-5,-7	70	10	17	27	126
6	57	M	AMMoL	47,XY,+21	88	14	8	22	40
7	29	M	AML	45,X,-Y	82	12	14	26	193
8	83	F	AML	47,XX,+9q-	51	4	13	17	47
9	63	F	AML	44,XX,+8,-12,-13,-14,t(2;5)	82	12	9	21	28
10	74	F	AML	48,XX,+3,+8	58	13	4	17	21
11	54	F	AML	46,XX,t(8;21)	89	2	2	4	40
12	51	F	AML	45,XX,-7	48	0	3	3	145
13	26	M	AML	46,XY,t(9;22)	68	1	2	3	198
14	55	M	AMMoL	46,XY,+8,-17	32	4	1	5	82
15	60	F	AML	47,X,-X,t(5;12),t(11;12), t(13;14),+16,+21	50	6	2	8	86
16	41	M	AML	-	0	2	6	8	571
17	73	M	AML	-	0	2	5	7	88
18	65	F	AMMoL	-	0	4	3	7	78
19	23	M	AML	-	0	3	4	7	180
20	46	M	EL	-	0	5	1	6	671
21	80	F	AMMoL	-	0	2	8	10	180
22	69	F	AMMoL	-	0	2	3	5	575
23	78	M	AMMoL	-	0	5	5	10	44
24	48	M	AML	-	0	3	1	4	154
25	49	M	AML	-	0	5	5	10	148

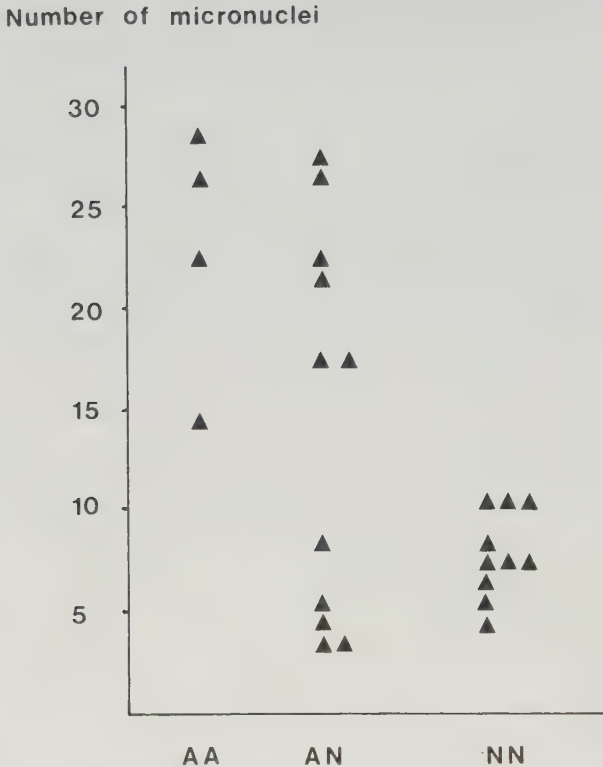


Figure 1 Relationship between number of micronuclei/2000 erythropoietic cells and cytogenetic pattern in 25 patients with ANLL.

Figure 1 shows the distribution of the number of micronuclei according to the cytogenetic pattern. The number of micronuclei varied between 14 and 28 (median 24) in AA patients and between 4 and 10 (median 7) in NN patients; there was no overlap between the two distributions. The AN group had an intermediate distribution ranging from 3 to 27 micronuclei, overlapping the NN and AA groups. However, as can be seen in Figure 2, the percentage of abnormal bone marrow metaphases and the number of micronuclei within the AN group are related. An increased frequency of abnormal metaphases seems to be associated with an increased frequency of micronuclei. If 2 patients (Cases No. 11 and 13), both of whom had a balanced translocation, are excluded the association is highly significant ($r = .83$, Spearman rank correlation coefficient).

Figure 3 shows a relationship between the number of micronuclei and survival time. Thus, patients with a small number of micronuclei have a better prognosis than patients with a large number. The Spearman rank correlation coefficient is $-.48$ ($0.02 > p > 0.01$, two tailed). When the total material is divided into 2 groups according to the number of micronuclei there is a striking difference in survival. The median survival time of patients with less than 10 micronuclei/2000 erythropoietic bone marrow cells is 148 days, whereas the median survival of patients with more than 10 micronuclei is only 34 days ($0.002 < p < 0.02$, Mann-Whitney two tailed U test). A similar difference in survival, 23 versus 180 days, is obtained when AA and NN patients are compared, irrespective of the number of micronuclei. The median survival

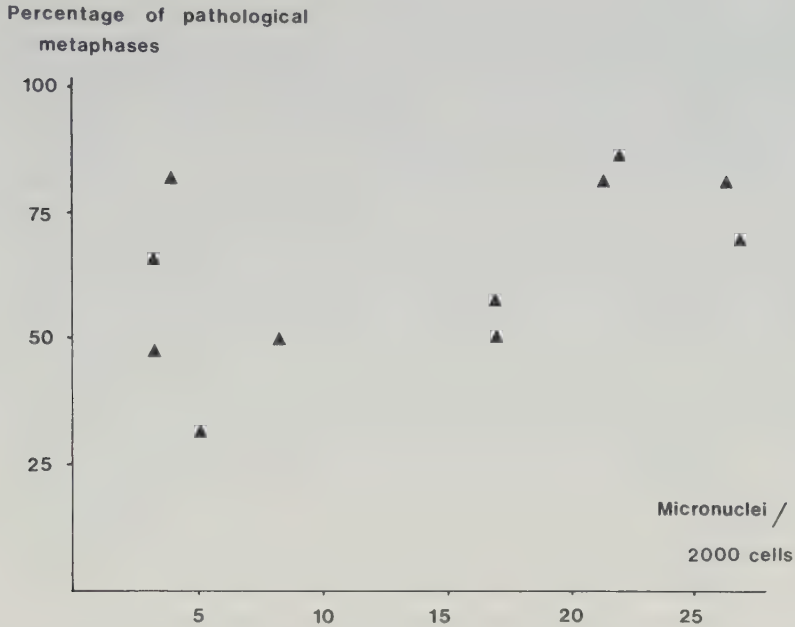


Figure 2 Relationship between percentage of abnormal bone marrow metaphases and number of micronuclei in 11 patients with a mixture of normal and abnormal metaphases (AN patients).

of AN patients is 82 days. As can be seen from Figure 2, the AN group seems to consist of two clusters: one with high numbers of micronuclei (range 17–27, median 22) and one with a low number of micronuclei (range 3–8, median 4). The median survival of the former group is 44 days and of the latter group 86 days.

DISCUSSION

It has been demonstrated that micronuclei or Howell–Jolly bodies are of nuclear origin [8–13]. They contain DNA and may originate as a result of different types of damage to the chromosomes, such as ionizing radiation, chemical agents, viruses, or other factors influencing the DNA metabolism [11, 12, 14–18]. The scoring of micronuclei is used, therefore, in the micronucleus test as a rapid and sensitive *in vivo* test for estimating the DNA-damaging effect of potential mutagenic/carcinogenic agents in animals [14]. Since bone marrow cells are needed for the test, it has only been used in a few studies on humans [11, 12, 15–18].

We examined bone marrow smears of 25 untreated patients with ANLL. The bone marrow karyotype at diagnosis had been determined previously using the trypsin–Giemsa banding technique. Micronuclei are particularly easy to distinguish in polychromatic erythrocytes, since young erythrocytes expel their nuclei shortly after completing the last erythroblastoid mitosis but retain their micronuclei: the Howell–Jolly bodies. In contrast to laboratory animals, human erythroblasts have visible cytoplasm. The micronuclei in these cells are also very easy to distinguish and thus may be used. In the present study we have calculated the total number of micronuclei in both erythroblasts and polychromatic erythrocytes. This has never been done in either animals or human beings. The Spearman rank correlation coefficient between

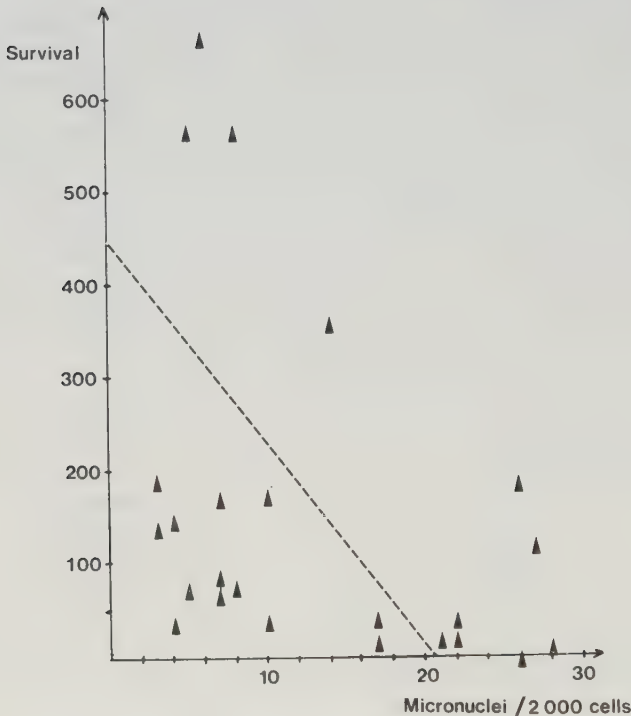


Figure 3 Association between number of micronuclei and survival in days in 25 patients with ANLL. The Spearman rank correlation coefficient is -0.48 ($0.01 < p < 0.02$, two tailed).

the frequency of micronuclei in the two types of cells was found to be 0.41 ($0.05 > p > 0.02$, two tailed), indicating that the mechanisms producing micronuclei in both cell types may be similar or related.

Our results show a relationship between the number of micronuclei in erythropoietic cells and the karyotype pattern of the bone marrow cells. Patients with a completely abnormal bone marrow karyotype had a significantly higher number of micronuclei than patients with only normal bone marrow metaphases. A similar relationship is apparently evident also in patients with a mixture of normal and abnormal bone marrow metaphases, since there is a relationship between the percentage of karyotypically abnormal cells and the number of micronuclei. It should perhaps be noted that this correlation is more evident if two patients, each with a balanced translocation, are excluded. It might be of interest that the two translocations were of a quite specific nature: $t(8;21)$, a translocation that has been demonstrated in about 10% of the patients with ANLL with aberrations [1, 19], and $t(9;22)$, i.e., the typical translocation producing the Philadelphia chromosome in chronic myeloid leukemia.

The underlying cause of the relationship between the frequency of micronuclei and the bone marrow karyotype in ANLL is obscure and difficult to explain. Micronuclei are believed to originate from different types of damage to the mitotic apparatus or the chromosomes, such as nondisjunction or breakage events, e.g., producing acentric fragments. The present findings would indicate that such events occur more commonly in a chromosomally abnormal bone marrow than in a bone marrow with

only normal diploid cells. In this way, once an abnormal karyotype has been established, new cells with chromosomal abnormalities would be produced at an accelerated rate. By selective pressure these mutational events will give rise to new abnormal clones, a well-known sequence of events in malignant cell populations. Whether the mutational events are induced actively or occur randomly is a question of great importance. It has recently been suggested that both types of changes may be involved in the neoplastic changes, since both are compatible with the nonrandom changes found in most neoplasms [19].

It has been demonstrated that the chromosomal abnormalities in leukemia are present in granulocytic, monocytic, erythropoietic, eosinophilic, and megakaryocytic cells [20–23]. Studies by Sakurai and Sandberg, discussed in detail in Sandberg [24], indicate that in erythroleukemia certain karyotypic changes are associated with morphologic abnormalities of the normoblastic cells. Because the micronuclei are studied in only erythropoietic cells it is tempting to speculate that in patients with a mixture of normal and abnormal bone marrow metaphases and a high number of micronuclei, chromosomal abnormalities are present in at least some of the erythropoietic cells. In contrast, patients with both normal and abnormal metaphases but with a low number of micronuclei would only have chromosomal abnormalities in their granulopoietic cells but not in the erythropoietic series. This occurrence remains to be clarified.

The presence or absence of chromosomal abnormalities in bone marrow cells of patients with ANLL has prognostic implications. Sakurai and Sandberg [2,3] first reported that patients with no normal bone marrow metaphases had a significantly shorter survival than those with at least one normal diploid bone marrow metaphase. This observation has subsequently been confirmed in several series of patients studied with modern banding techniques [1, 4, 5]. In a recent study from our laboratory [5] the median survival of patients with exclusively normal chromosomes (NN) was 8.5 months, whereas patients with abnormal chromosomes (AA) lived only 1.5 months. In the present series the corresponding figures were 6 months and less than 1 month. It is of particular interest that similar differences in survival are obtained when patients with high and low numbers of micronuclei are compared, irrespective of the chromosomal patterns. Thus, patients with less than 10 micronuclei/2000 erythropoietic cells have a median survival of 5 months compared to a median survival of 1 month in patients with more than 10 micronuclei/2000 erythropoietic cells. The number of micronuclei furthermore gives an opportunity to subdivide the AN group. Although the number of patients is small, it is interesting to note that AN patients with fewer than 10 micronuclei/2000 erythropoietic cells have a median survival of 3 months, whereas AN patients with more than 10 micronuclei/2000 erythropoietic cells have a median survival of 1.5 months. As can be seen in Figure 3 the relationship between survival and the frequency of micronuclei is evident also for each individual case in the total material.

Since there is an association between the frequency of micronuclei and the chromosomal pattern, it is not possible to state whether the prognosis is determined by the number of micronuclei or the chromosomal pattern or both. In any case, the results of micronuclei scoring correlate well with the more laborious assay based on a direct chromosome analysis. Examining one individual by counting the number of micronuclei takes about 2 to 4 hours, which is a rather short time compared with bone marrow karyotype analyses. For this reason, the micronuclei test may become a valuable aid in the evaluation of patients with ANLL. It should be mentioned, however, that the prognostic value of the test is restricted to untreated patients, since cytostatic treatment will increase the number of micronuclei in bone marrow cells [15–17].

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Brief report

The interrelations of micronuclei, chromosomal instability, and mutational activity in acute non-lymphocytic leukemia—A hypothesis

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In a recent study (HÖGSTEDT et al. 1981), we found a relationship between the number of micronuclei and the presence of chromosomal abnormalities in the bone marrow cells of patients with acute non-lymphocytic leukemia (ANLL). Thus, the number of micronuclei was significantly higher in patients with abnormal metaphases than in those with normal metaphases; in patients with a mixture of normal and abnormal bone marrow metaphases, the frequency of abnormal metaphases was correlated to the number of micronuclei. Furthermore, a significant negative correlation prevailed between the number of micronuclei in erythropoietic bone marrow cells and the survival of the patients; this was so, irrespective of the karyotypic bone marrow pattern.

In this paper we would like to discuss another aspect, namely the interrelations of micronuclei, chromosomal instability, and mutagen activity. The possible association between chromosomal instability and mutagen activity has been discussed for several years on the basis of work on cultivated human cells and on cells of plants with constitutional chromosome anomalies (see below). No such information is available, however, regarding acquired clonal chromosomal aberrations of somatic human cells in vivo.

ISING (1962, 1969) was the first to establish that somatic karyotypic anomalies may predispose to structural chromosomal variation. In *Cyrtanthus*, a karyotypically variable genus of the Amaryllidaceae family, Ising recorded the incidence of spontaneous structural aberrations, translocations, pericentric inversions, different types of deficiency, and found that in aneuploid individuals this incidence was significantly higher than in euploids.

In man, similar studies have been performed on individuals with constitutional chromosomal abnormalities. The results of SASAKI and TONOMURA (1969) and SASAKI et al. (1970) are of special in-

terest. They found that lymphocytes of trisomic or partially trisomic patients (excess of chromosome material) were twice as sensitive as those of normal diploid persons to the induction by γ -irradiation of exchange type chromosome aberrations (dicentric and rings). Similar sensitivity to X-irradiations has been reported in patients with Down's syndrome and other congenital chromosomal aberrations (LAMBERT et al. 1976; SEABRIGHT 1976; GOH et al. 1978).

HIGURASHI et al. (1973) have shown that lymphocytes from patients with regular trisomy 21 had a significantly higher frequency of chromosomal damage after measles infection than a control group with normal karyotype. As a matter of fact, the patients with Down's syndrome had twice as many aberrations as the controls even before the infection and this difference became even more pronounced after infection. SCHULER et al. (1972) found that lymphocytes from four patients with Down's syndrome exposed in vitro to an alkylating agent displayed significantly more structural chromosomal changes than normal cells. Similarly, COUNTRYMAN and HEDDLE (1976) found that irradiation of cultured lymphocytes from patients with Down's syndrome produced twice as many micronuclei as irradiation of lymphocytes from normal individuals. Also, an increased frequency of sister chromatid exchanges was found in lymphocytes from Down's syndrome patients after exposure to both 50 and 100 rads, whereas normal controls showed an elevated rate only after exposure to 100 rads (CROSSEN and MORGAN 1980). An interesting observation in this context is that fibroblasts from patients with Down's syndrome or other constitutional chromosome abnormalities have an increased susceptibility to undergo transformation by SV 40, adenovirus type 7 and polio virus type 3 (TODARO and MARTIN 1967; STENRAM et al. 1969). Also, recent studies have indicated that there may be differences in DNA-repair syn-

thesis between patients with Down's syndrome and controls (LAMBERT et al. 1976).

The results mentioned above clearly suggest a close correlation between certain types of constitutional karyotypic abnormalities and the production of structural chromosome aberrations and micronuclei. These results, no doubt, resemble those we obtained when studying acquired chromosome aberrations of bone marrow cells in vivo (HÖGSTEDT et al. 1981). We therefore speculate that similar mechanisms may operate in the formation of aberrations in both constitutional and acquired conditions. In the investigations by ISING (1962, 1969), HIGURASHI et al. (1973), and by ourselves the aberrations appear to occur spontaneously; whereas in the investigations of, e.g., SASAKI et al. (1970); SCHULER et al. (1972); COUNTRYMAN and HEDDLE (1976); and CROSSEN and MORGAN (1980) they occurred after exposure to irradiation or chemicals. However, such exposures probably have the effect just to make existing differences more clear.

The association between micronuclei and acquired chromosomal abnormalities is interesting from different aspects. It has been shown in animals and man that micronuclei are sensitive indicators of the mutagenic/carcinogenic effects of ionizing radiation and also different types of chemical agents (SCHMID 1975). Since micronuclei are, thus, closely related to mutational events, it is tempting to speculate that the rate of such events is higher in patients with ANLL and an abnormal bone marrow karyotype than in those with no abnormalities. This indicates that once an abnormal karyotype has been established, new cell clones with additional chromosomal abnormalities will be produced at an accelerated rate. In fact, this phenomenon has been demonstrated in experimental malignant tumors (MITELMAN 1972). Such an increased mutational activity may be the mechanism underlying the poor prognosis of leukemic patients with abnormal karyotype (SAKURAI and SANDBERG 1976; First International Workshop on Chromosomes in Leukemia 1978; GOLOMB et al. 1978; MITELMAN et al. 1978) and, possibly, also explain why patients with certain constitutional chromosomal aberrations have an increased risk of developing malignant disorders, e.g., acute leukemia in trisomy 21 (ref. in SANDBERG 1980).

Increased susceptibility and/or decreased resistance of such cells to chromosome-damaging effects of environmental agents are possible mechanisms. If so, the study of micronuclei may be a useful assay system in man to detect individ-

uals with a higher susceptibility to environmental hazards, e.g., occupational exposure to potential mutagenic/carcinogenic agents.

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Micronuclei and chromosome aberrations in bone marrow cells and lymphocytes of humans exposed mainly to petroleum vapors

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Several cytogenetic parameters, i.e. chromosome aberrations in bone marrow cells and in peripheral lymphocytes as well as micronuclei in erythropoietic bone marrow cells, were studied in 16 tank cleaners exposed to organic solvents and other petroleum products and sediments containing heavy metals, such as lead, mercury, arsenic and cadmium. Micronuclei and chromosome breaks in peripheral blood lymphocytes proved significantly more common in tank cleaners than in controls. The data indicated an exposure-connected relationship, suggesting that assessment of micronuclei in bone marrow cells might be a useful method for detecting cytogenetic effects in humans chronically exposed to chemical agents. In this respect both erythroblasts and polychromatic erythrocytes could be studied.

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One possibility of assessing mutagenic/carcinogenic effects of environmental agents in humans is the examination of bone marrow cells, which gives an excellent chance to study both erythropoietic cells in interphase (micronuclei) and erythropoietic and myelopoietic cells in metaphase (chromosome aberrations). Although increase of micronuclei and/or chromosomal aberrations has been reported in bone marrow cells of humans exposed to ionizing radiation and cytostatic drugs (FLIEDNER et al. 1964; ISHIHARA et al. 1973; AWA 1974; GOETZ et al. 1975, 1976; KAMADA and UCHINO 1976; KROGH JENSEN and HÜTTEL 1976; KROGH JENSEN and NYFORS 1979) this approach has been used only to a very limited extent. Exposure of humans to organic solvents and/or petroleum products has been associated with chromosomal damage in peripheral blood lymphocytes (FORNI et al. 1971; FUNES-CRAVIOTO et al. 1977; FREDGA et al. 1979) and with malignant disorders (INFANTE et al. 1977; BRANDT et al. 1978; OLSSON and BRANDT 1979; THOMAS et al. 1980). Further studies are urgent as such exposure is very common in industrialized societies.

We here report on a study of micronuclei and chromosome aberrations in bone marrow cells as well as in peripheral blood lymphocytes of humans chronically exposed to organic solvents and/or

petroleum products as well as to some heavy metals.

The following abbreviations will be used: PBL = peripheral blood lymphocytes; BMC = bone marrow cells; EB = erythroblasts; PCE = polychromatic erythrocytes.

Material

Exposed subjects

16 males (aged 27–58, median 35 years) working as tank cleaners in the same enterprise were studied. Physical and routine laboratory examinations revealed nothing remarkable: the blood hemoglobin value, white blood cell count and erythrocyte sedimentation rate were normal in all. None showed proteinuria or glucosuria.

They were interviewed regarding their occupational and medical history, especially concerning viral infections, use of alcohol and of drugs, smoking habits, exposure to ionizing radiation and heavy metals. Ten of the men smoked (10–30, median 15 cigarettes/day). Five were ex-smokers, who had all stopped more than three years ago. Only one had never smoked.

One worker had had an abdominal X-ray

examination because of an accident 9 months before the investigation. This worker as well as another one had minor alcoholic problems. No other factors known to be capable of damaging chromosomes were found.

All the men had been employed as cleaners for 4 months to 20 years (median 7 years). All but one had been doing various types of work connected with the cleaning of the tanks. These may be divided into 5 main tasks (a–e, below). The distribution of the working hours among these tasks was calculated from the daily reports of ten workers for a ten-month period.

a. Oil tanks.—(0–81 %, median 46 % of total working hours). The tanks were of different sizes (2–10,000 m³) and contained different types of light or heavy oils. When the emptied tanks were to be cleaned, the last portion of the oil was removed by hand. Tanks containing heavy oils were cleaned with a solution of white spirit, xylene and a non-ion active detergent, which was sprayed on to the walls of the tank. The temperature in this type of tank was sometimes as high as 30–50°C. Apart from rubber boots, no personal protecting equipment was used. Respirators with coal filter were available but only occasionally used.

Estimations of the air-levels of total hydrocarbons in one emptied light oil tank were made. Direct infrared spectrophotometric readings ranged from 100–300 ppm.

b. Petrol tanks.—Only four workers had been cleaning petrol tanks during the last ten months, and then for only 1–7 % of their working hours. When these large depot tanks had been emptied, there was still some sediment on the bottom. For the last few years, the men had used personal protecting equipments, such as compressed air respirators, and rubber gloves and boots, but just an ordinary cotton overalls. This type of work involved skin contact with petrol and organo-lead compounds.

c. Solvent tanks.—(0–11 %, median 2 % of working hours). These tanks were relatively small and contained organic solvents such as white spirit, xylene, toluene, acetone, and ethylacetate. Tanks containing formaldehyde and phenol were also cleaned. The solvents were often contaminated with other chemicals. Protective rubber dress and compressed air respirator were available, but the manhole was often so small as to prevent the use of this equipment.

d. Chemical wastes and sewage content.—(1–100 %, median 12 % of total working hours). Sediments were sucked through a tube to a tank on a lorry with a diesel engine. The sediments were both organic from sewage systems of ordinary houses and chemical from factories, containing inorganic salts of lead, arsenic, mercury and cadmium as well as other non-specified chemical compounds. This type of work was performed without any personal protective equipment. Skin contact was common.

e. Oil discharge.—(0–16 %, median 14 % of working hours). Oil that had leaked out onto soil or into water was sucked or removed with a solvent containing white spirit, xylene and a detergent. No personal protective devices were used.

a–e. All individuals were divided into two groups. A highly exposed group consisted of 9 men, who had been exposed for more than 6 months and who were still working (8) or had stopped one year previously (1). A less exposed group consisted of men who had either stopped work for more than two years (3), who had worked for less than 6 months (2), or only with sewage sediments (1), or who had worked partly as an attendant (1).

Controls

The controls consisted of two groups sampled in close connection with this study. Six paper factory workers, not occupationally exposed to chemicals, were studied three months before the exposed subjects in this study (HÖGSTEDT et al. 1979a). 10 bus drivers were examined 6 months after the exposed subjects (HÖGSTEDT et al. 1979b). The same background data were obtained on the controls as on the subjects exposed.

Methods

Peripheral blood

1. Chromosome aberrations in lymphocytes

A standard microculture technique was used. The cultivation time was 72 h. Preparations were coded and analyzed by two observers without knowledge of the exposure (see Discussion). 200 metaphases were scored from each individual and the aberrations were recorded according to the classification system recommended by EVANS et al. (1980). The chromosomal aberrations recorded

included chromatid breaks, chromatid interchanges, acentric fragments, ring chromosomes, dicentric chromosomes and pericentric inversions. Gaps, both chromatid and isochromatid, were not included. Agreement between the two observers was required for scoring a cell as abnormal; observed cells were never rejected. Samples from exposed and control individuals were prepared and analyzed in the same way.

2. Blood lead analyses

Venous blood was obtained at the same time as the cytogenetic samples. Lead was determined by atomic absorption spectrophotometry (SCHÜTZ and SKERFVING 1976). The limit of detection was 0.01 mg/l, and the error of the method was 4 %. Repeated cooperative Scandinavian studies have shown a good accuracy of the method even at low blood levels.

Bone marrow

1. Micronuclei

Bone marrow cells were obtained by sternal puncture at the same time as the blood samples. The marrow cells were prepared according to the micronucleus test method described by SCHMID (1975) as modified by JENSSEN and RAMEL (1976). The cells were suspended in a mixture of equal parts of isotonic phosphate buffer, (pH 7) and fetal calf serum. After vigorous shaking, the cells were pelleted and the supernatant was removed. The pellet was resuspended in a few drops of buffer and calf serum solution. The cells were smeared on glasses, air dried, and then smears were stained with May-Grünwald-Giemsa's stain. Only interphase cells were analyzed. The micronucleus in a polychromatic erythrocyte (PCE) was defined as a rounded, bluish or almost black, sharply outlined structure, about 1 μ m across. The micronucleus in an erythroblast (EB) was somewhat larger and of the same colour as the main nucleus. For each individual, 1,000 PCE and 1,000 EB, mostly of the polychromatic type, were examined by one observer, who was not aware of the exposure data.

2. Chromosome aberrations

Bone marrow chromosomes from 15 tank cleaners were analyzed; one case had to be excluded since too few mitoses were found. No appropriate controls were available. The bone marrow cells used

for chromosome studies were obtained from the same sample as the cells for the micronucleus test. The cells, suspended in isotonic phosphate buffer (pH 7) were centrifuged a few hours after collection, and incubated over night in McCoy's medium without PHA stimulation. Otherwise, preparations were made in the same manner as the PBL. In order to identify break points in individual chromosomes, the bone marrow cells were stained with the trypsin-Giemsa banding technique.

50 mitoses from each individual were analysed without knowledge of exposure. The aberrations were classified according to the same system as that used for the PBL. In addition, 5–11 mitoses from each individual were photographed and karyotyped in detail.

Statistical methods

When comparing the three different exposure groups (tank cleaners with high and low exposure, and controls) the "Kruskall-Wallis test of ordered alternatives" (BRADLEY 1968) was used; for comparison of two groups (tank cleaners versus controls), the "Mann-Whitney U test"; and for analysis of correlation, the "Spearman rank correlation test" (SIEGEL 1956). Only two-tailed P-values are given. "Statistical significance" denotes $P < 0.05$.

Results

The individual values noted for each parameter studied are summarized in Table 1. There were significant and exposure-associated differences between the groups (tank cleaners with high and low or previous exposure, and controls) in frequency of chromosome aberrations in PBL (Fig. 1; $P < 0.02$), frequency of micronuclei in EB and PCE (Fig. 2; $P < 0.05$ and $P < 0.01$, respectively) and blood lead levels (Fig. 3; $P < 0.01$). There were no significant differences between the two exposed groups regarding the frequency of chromosome aberrations in BMC (Fig. 4).

When smoking tank cleaners were compared to smoking controls, there were significant differences in chromosome aberrations in PBL ($P < 0.03$; $n_1 = 11$, $n_2 = 8$), frequency of micronuclei in PCE ($P < 0.03$; $n_1 = 11$, $n_2 = 5$), and blood lead levels ($P < 0.04$; $n_1 = 11$, $n_2 = 5$), but not regarding the frequency of micronuclei in EB ($P < 0.11$; $n_1 = 11$, $n_2 = 5$).

Table 1. Summary of exposure-related and cytogenetic parameters in 16 tank cleaners and 16 controls

Case No.	Exposure-related parameters			Cytogenetic parameters			
	Exposure group	Smoker	Blood lead (mg Pb/l)	Bone marrow cells			Lymphocytes
				No. of micronuclei in erythroblasts (%)	polychromatic erythrocytes (%)	Chromosomal aberrations (%)	Chromosomal aberrations (%)
1	High	Yes	0.10	1	8	2	6.5
2		"	0.10	3	15	8	3.5
3		"	0.10	8	31	10	2
4		"	0.15	4	14	4	6.5
5		No	0.10	0	10	ND	ND
6		Yes	0.08	1	5	12	2.5
7		"	0.18	6	4	10	4.5
8		"	0.09	2	8	4	5
9		"	0.07	4	19	8	2.5
10	Low	"	0.08	5	4	8	4.5
11		No	0.09	8	21	6	3
12		"	0.09	1	4	4	3
13		"	0.08	0	2	10	6.5
14		Yes	0.10	4	6	14	3.5
15		No	0.06	4	8	0	3
16		Yes	0.10	2	4	2	5.5
17		Control	0.05	5	13	ND	3
18		"	0.06	1	0	"	1.5
19	Control	No	0.06	0	0	"	2
20		"	0.03	0	1	"	1.5
21		"	0.03	0	6	"	0.5
22		"	0.07	0	2	"	1.5
23		Yes	0.08	1	1	"	4.5
24		"	0.10	0	2	"	3.5
25		No	0.07	1	3	"	4
26		Yes	0.07	2	4	"	3
27		No	ND	ND	ND	"	3
28	Control	Yes	"	"	"	"	2.5
29		"	"	"	"	"	0.5
30		"	"	"	"	"	0.5
31		No	"	"	"	"	2
32		"	"	"	"	"	4.5

ND = Not done

There was a significant correlation between the blood lead values and the frequency of chromosome aberrations in PBL (Fig. 5; $r_s=0.65$; $P<0.001$, $n=25$), but not between the lead values and other cytogenetic parameters, i.e. micronuclei in EB ($r_s=0.32$; $P<0.10$, $n=26$) and PCE ($r_s=0.36$; $P<0.07$, $n=26$) and chromosome aberrations in BMC ($r_s=0.07$; $P>0.10$, $n=15$).

A significant correlation was found between the frequencies of micronuclei in EB and PCE in the combined material of tank cleaners and controls (Fig. 6; $r_s=0.64$, $P<0.001$, $n=26$). Otherwise, there were no significant correlations between the cytogenetic parameters, i.e. chromosome aberrations in PBL versus micronuclei in EB ($r_s=0.21$, $n=25$), micronuclei in PCE ($r_s=0.12$, $N=25$) and chromosome aberrations in BMC ($r_s=-0.32$,

$n=15$), or chromosome aberrations in BMC versus micronuclei in EB ($r_s=0.17$, $n=15$) and micronuclei in PCE ($r_s=-0.09$, $n=15$).

Discussion

Cytogenetic effects of exposure

The tank cleaners had been exposed to different petroleum products, organic solvents, and heavy metals. Some of these chemicals have a known or suggested mutagenic/carcinogenic activity (FORNI et al. 1971; FUNES-CRAVIOTO et al. 1977; HERNBERG 1977; NORDENSON et al. 1978a, b; FREDGA et al. 1979). Our findings indicate that even in an "emptied" oil tank an unprotected worker may

Frequency of chromosomal aberrations (%)

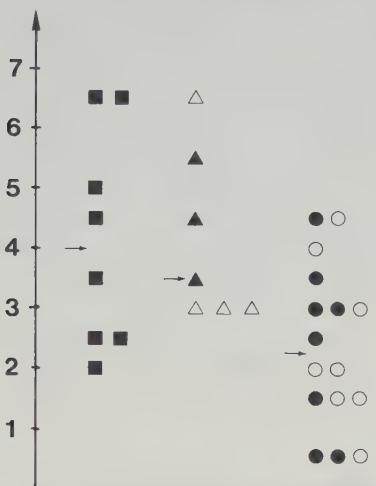


Fig. 1. Frequency of chromosomal aberrations in peripheral blood lymphocytes in tank cleaners with high (squares) and low (triangles) exposure and controls (circles). Smokers closed, non- and ex-smokers open symbols. Median values are indicated by arrows.

Frequency of micronuclei (‰)

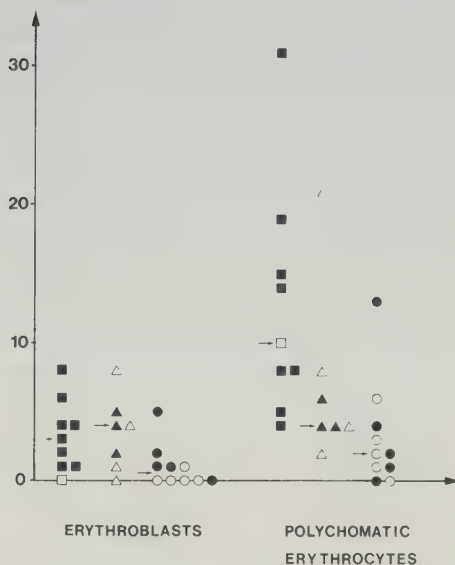


Fig. 2. Frequency of micronuclei in erythroblasts and polychromatic erythrocytes in tank cleaners with high (squares) and low (triangles) exposure and controls (circles). Smokers closed, non- and ex-smokers open symbols. Median values are indicated by arrows.

suffer an intense exposure at or above the threshold limit values for different petroleum vapors (Limit Values 1978). Also poor working conditions were present regarding personal hygiene, e.g., no possibility of hand-washing before eating, smoking etc. Closer estimation of the exposure was not possible, our measurements being few, the types of work varying, and respirators occasionally being used.

The statistical evaluations of the cytogenetic effects in the three groups studied showed significant exposure-related differences for each cytogenetic parameter (chromosome aberrations in PBL and micronuclei in EB and PCE). This exposure-related cytogenetic effect lends support to a causal relationship.

The blood lead values showed a similar pattern of variation as the cytogenetic parameters. There was also a significant correlation between the blood lead values and the chromosome aberrations in PBL. A similar correlation has previously been reported among bus drivers (HÖGSTEDT et al. 1979b). Since it is unlikely that lead has a mutagenic/carcinogenic effect (O'RIORDAN and EVANS

1974; HERNBERG 1977; NORDENSON et al. 1978b), we prefer to look upon this substance as an index of exposure to other compounds with more evident mutagenic/carcinogenic effects. It should be noted that the blood lead levels in the present study, in spite of the group differences, were all within the normal range of a Swedish population.

A few remarks should be made on the comparability of the groups. The controls were sampled partly before and partly after the tank cleaners. Cell cultivation conditions for PBL and BMC may differ slightly from time to time. The samples were analysed for chromosomal aberrations by two observers. One screened the major part of the material, the other, some of the controls (bus drivers). All aberrations were, however, agreed upon by the two observers together. As to confounding factors, smoking is one possibility. However, the differences in most cytogenetic parameters, as well as blood lead values, were still obvious and significant when smoking tank cleaners and smoking controls were compared. Also after correction

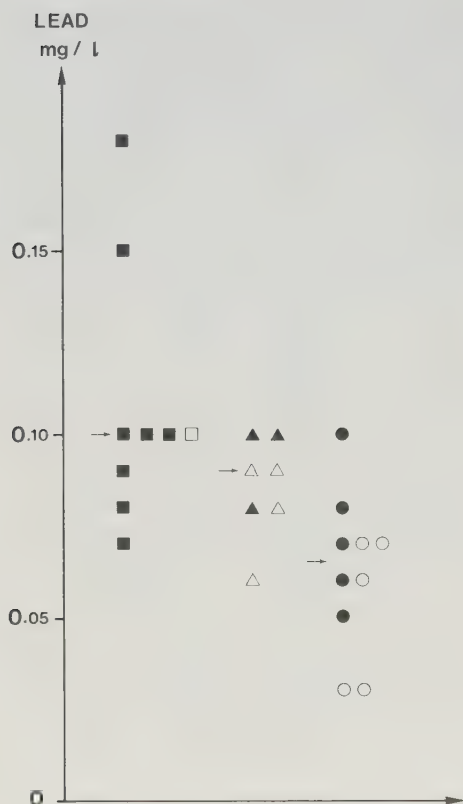


Fig. 3. Blood lead values in tank cleaners with high (squares) and low (triangles) exposure and controls (circles). Smokers closed, non- and ex-smokers open symbols. Median values are indicated by arrows.

for smoking habits, in an analysis of variance, the differences remained essentially unchanged.

Relations among cytogenetical parameters

Despite the above-mentioned significant dose-related differences found in the three exposure groups, regarding chromosome aberrations in PBL and micronuclei in EB and PCE, there was a remarkable lack of individual correlations among the different cytogenetic parameters. This may, however, be explained as follows: (1) The cells studied represent completely (PBL vs. BMC) or partly different cell types (erythropoietic in EB and PCE vs. myeloid and erythropoietic in BMC). (2) Micronuclei are considered to be the result of

Frequency of chromosomal aberrations (%)

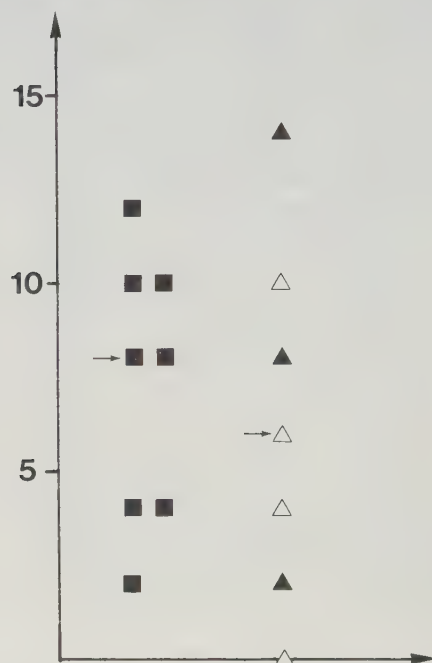


Fig. 4. Frequency of chromosome aberrations in bone marrow cells in tank cleaners with high (squares) and low (triangles) exposure. Smokers closed, non- and ex-smokers open symbols. Median values are indicated by arrows.

non-disjunction and breakage events producing acentric fragments, but not all chromosomal abnormalities will form micronuclei. (3) The aberrations recorded in metaphases from PBL cultivated for 72 h represent a mixture of cells in first, second and third mitoses. Since many aberrations are certainly lethal to the cell, metaphases representing second and third mitoses may have fewer aberrations than first-division metaphases. However, the metaphases of bone marrow cells all represent first-division cells. Also, only G_0 cells are assayed in a peripheral blood investigation; cells in other stages of the cycle may be more sensitive. (4) The lack of correlation between chromosomal breakages in BMC and PBL may also be due to the small number of cells studied in BMC and/or methodological differences.

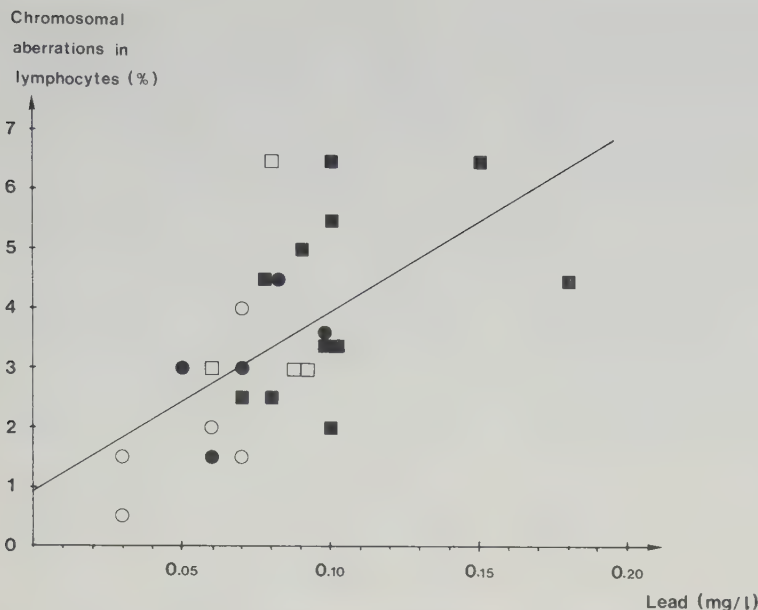


Fig. 5. Relation between the frequency of chromosome aberrations in lymphocytes and blood lead values in tank cleaners (squares) and controls (circles). Smokers closed, non- and ex-smokers open symbols. The "Spearman rank correlation coefficient" is 0.65; $P < 0.001$, two-tailed.

No systematical study has been performed on the relationship between chromosome aberration frequencies in lymphocytes and bone marrow cells. In general, a lower frequency of chromosomal aberrations in bone marrow cells has been reported (KNUUTILA et al. 1977; BRÖGGER 1979). This may be due to technical reasons—bone marrow chromosomes are usually short, so that gaps and certain types of breaks are not easily detected and may, therefore, have been overlooked—or the sensitivity of the two materials may be different. In the present study, there were twice as many aberrations in BMC as in PBL. Since no control individuals were available as regards BMC, it is not known whether this is a real difference. However, our results are in accordance with the findings of KROGH JENSEN and NYFORS (1979).

The micronucleus test has been extensively used in mice. In this species, only PCE are suitable since EB have little or no visible cytoplasm. In man, EB has sufficient cytoplasm to harbour micronuclei and can therefore be used. The highly significant correlation demonstrated in the present

study, between micronuclei in EB and PCE, also implies a close relationship between the two cell types.

Analysis of micronuclei has earlier proved useful for assessing the effects of short time exposure to mutagenic agents in man (FLIEDNER et al. 1964; GOETZ et al. 1975, 1976, KROGH JENSEN and HÜTTEL 1976; KROGH JENSEN and NYFORS 1979) and in experimental animals (SCHMID 1975; JENSEN and RAMEL 1980). This study shows that the technique can also be useful when studying the effects of chronic exposure of man to chemicals. The micronucleus method has one major drawback: the need for bone marrow sampling. On the other hand, it may offer several advantages over the often used method of examination for chromosomal aberrations in PBL: First, the rapid and rather uniform turnover of the erythropoietic cells *in vivo* may be an advantage compared to the slow and varying turnover of the PBL, micronuclei thus probably being a better index of recent cytogenetic damage. Second, erythropoietic cells constitute another cell type, possibly with different susceptibility than BMC (partly) or PBL. Third, the study of

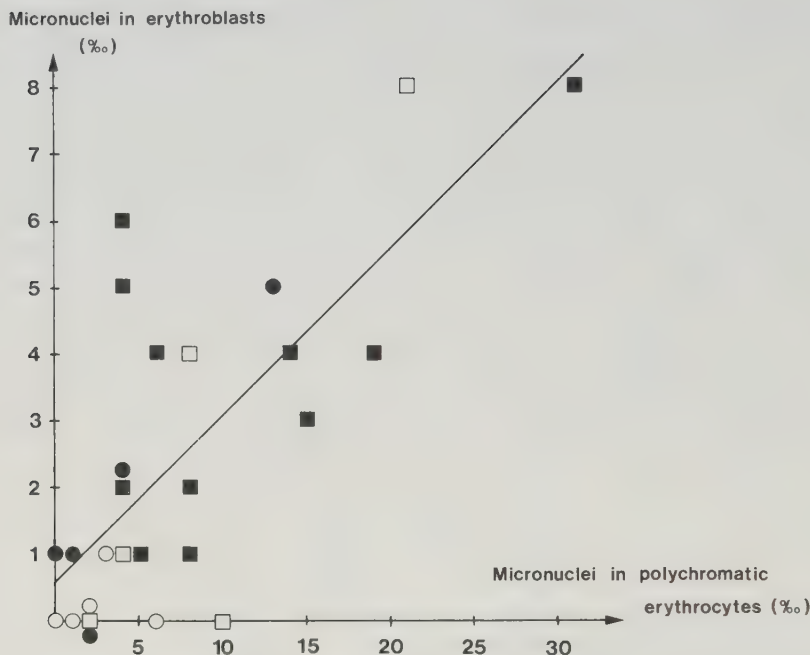


Fig. 6. Relation between the frequency of micronuclei in erythroblasts and polychromatic erythrocytes in tank cleaners (squares) and controls (circles). Smokers closed, non- and ex-smokers open symbols. The "Spearman rank correlation coefficient" is 0.64; $P < 0.001$, two-tailed.

micronuclei reflects the situation *in vivo* since it does not include cell cultivation, which may be a source of error. Fourth, the analysis is much simpler, faster and less subjective.

The facts mentioned above may explain why there were more evident differences in the frequency of micronuclei than chromosomal aberrations in PBL between the two groups of tank cleaners and controls. Another important factor may be that the less exposed group of tank cleaners contained some workers who had not been exposed for the last two years; since micronuclei may be a more dynamic index of cytogenetic effects, it is reasonable that they show more pronounced differences.

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Chromosome aberrations and micronuclei in bone marrow cells and peripheral blood lymphocytes in humans exposed to ethylene oxide

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Twenty-eight individuals occupationally exposed to ethylene oxide have been compared with 20 controls regarding cytogenetic effects in peripheral blood lymphocytes and in bone marrow cells. The exposure levels of ethylene oxide were estimated and none of the individuals had, during the last 2.5 years, been exposed to workroom air concentrations exceeding 1 ppm. The exposed persons showed significantly increased levels of chromosome aberrations in lymphocytes (about 50 % increase of mean) and of micronuclei in erythroblasts and polychromatic erythrocytes (about 100 % and 300 % increase of mean, respectively) when compared with controls. However, sister chromatid exchanges and micronuclei in lymphocytes did not show any significant effect of exposure. A significant effect of smoking on micronuclei in erythroblasts and lymphocytes was found. There were several statistically significant, positive correlation coefficients among the different cytogenetic parameters.

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Ethylene oxide has alkylating properties that are due to the epoxide structure of the molecule. It reacts with DNA primarily at the N-7 position of guanine to form N-7-hydroxyethyl-guanine (BROOKES and LAWLEY 1961; EHRENBERG et al. 1974). It may also have affinity to other macromolecules (e.g., enzymes) in the body. Ethylene oxide has an inhibitory effect on the human DNA-repairing mechanism both in vitro and in vivo (PERO et al. 1981; PERO et al. 1982). It has a well documented ability to cause mutations in different test systems; in bacteria (PFEIFFER and DUNKELBERG 1980; RANNUG et al. 1976), in plants (EHRENBERG et al. 1956), in *Drosophila* (RAPOPORT 1948) and in mammals (APPELGREN et al. 1978; CONAN et al. 1979; EMBREE and HINE 1975).

Already in 1961, Ehrenberg and Hällström (KALLINGS 1967) obtained indications that persons highly exposed to ethylene oxide had an increased frequency of chromosomal aberrations in lymphocytes, but they investigated only a few metaphases in a small number of persons. The same results have recently been obtained by ABRAHAMS (1980). Evidence has also been published indicating that ethylene oxide may cause haematological and

other types of malignancies in animals and man (DUNKELBERG 1979; HÖGSTEDT et al. 1979a, b).

The aims of the present study were to clarify whether ethylene oxide really causes chromosomal damage in man as measured by several cytogenetic methods, i.e. chromosomal aberrations, sister chromatid exchanges, and micronuclei in peripheral blood lymphocytes, and also chromosomal aberrations and micronuclei in bone marrow cells. The relation between levels of ethylene oxide in workroom air and the cytogenetic effects, and the association between the different cytogenetic parameters have also been studied.

Materials and methods

1. Interviews, physical examination and biological sampling

All subjects in this investigation, exposed as well as controls, were examined and interviewed by the same persons about diseases, drug intake, exposure to ionizing radiation or compounds with known or suspected mutagenic ability. Blood

samples from both the exposed workers and the controls from factory I were obtained on three consecutive days (October 1978) and from factory II five months later (March 1979) on two consecutive days. Bone marrow specimens of the workers in factory I were sampled in October 1978 and of the controls in May 1978.

2. Analysis of ethylene oxide air concentrations

Workroom air specimens were collected in whole-glass syringes (50 ml) during 30 min or 2 h by personal and stationary area sampling. The air samples were analyzed on a AID Portable Gas Chromatograph Model 514 equipped with a flame-ionization detector using a 2 m, 1/8" stainless steel column packed with Purepac Q, 100–120 mesh. The carrier gas was N₂, flow rate 20 ml/min; column temperature 150°C. The detection limit was 0.2 ppm but, in the measurements during 1975, levels below 1 ppm were unfortunately denoted in the protocol by a value of zero. Therefore, in Table 1, the exposure levels in some cases are given as a range, where the limits are calculated from the detection limits (1.0 and 0.2 ppm) and from zero ppm. Time-weighted averages (TWA) of exposure were calculated.

3. Working conditions and subjects studied

3.1 Factory I

Working conditions. — Medical equipment wrapped in plastic foil was treated in two sterilizers with a mixture of 50 % ethylene oxide and 50 % methyl formate. After treatment the sterilizers were air-flushed several times. They were opened for a period of 30 min, twice a day. The sterilizers were unloaded and the equipment placed in a ven-

tilated stockroom for 24 h, after which it was packed in paper boxes in a special workroom. The sterilizing personnel were exposed to ethylene oxide when loading and unloading the sterilizers (Table 1; momentary peak levels 52 ppm), which in all took 1–2 h a day. During the rest of the working day they were not exposed. One year before the study the sterilizing personnel began to wear breathing protective devices while the sterilizers were open. During the sterilization process ethylene oxide penetrates the plastic covers of the equipment. For several days afterwards it diffuses and thus pollutes the air of the stockroom (Table 1) and of the packingroom. A laboratory assistant worked in the stockroom for approximately 1 h a day. An attendant was exposed to ethylene oxide for a few hours a week while changing the gas cylinders. Between measurements in June and December 1975 the working routines were changed and consequently the levels decreased considerably (Table 1). No further changes were made until biological sampling in October 1978.

Exposed persons. — The exposed persons (Table 2) consisted of 18 individuals (17 females and 1 male) aged 19 to 55 (mean 37) years. They had been exposed to ethylene oxide for 0.5–8 (mean 3.2) years. Four were sterilizing and 12 were packing personnel, 1 was a laboratory worker, and 1 a male attendant.

Taking into consideration levels of ethylene oxide, working routines and use of protective devices it may be concluded that at the time of biological sampling in October 1978, all subjects were exposed to 8 h TWA levels below 1 ppm. Until three years earlier, the exposure levels were higher, though probably not exceeding 5 ppm. Five persons had had various X-ray examinations within one year before the investigation. Four persons took hormones as contraceptives or against

Table 1. Ethylene oxide levels (ppm, TWA) for different types of work operations

	Factory I				Factory II	
	June, 1975		December, 1975		May, 1978	
	Personal sampling	Area sampling	Personal sampling	Area sampling	Personal sampling	Area sampling
<i>Sterilization room</i>						
sterilizer open	29	28	7.6	5.0		
sterilizer closed	8.9	6.4	1.3	0.6	2.4*	1.3*
<i>Stockroom</i>	—	12	—	1.8	—	0.8
<i>Packing room</i>	4.5	4.4	0.3–1	0.1–1	0.1	—

* Sampling during total shifts

Table 2. Background data and result of cytogenetic analyses in persons exposed to ethylene oxide and controls (ND = not done)

No.*	Age	Sex	Smoking	Peripheral blood lymphocytes					Bone marrow cells		
				Cells with chromosomal aberrations			SCE (Mean No./ cell)	Micro- nuclei (%)	Chromo- somal aberra- tions (%)	Micronuclei in	
				Breaks (%)	Gaps (%)	Total (%)				Erythro- blasts (%)	Polychromatic erythrocytes (%)
1	58	F	no	2.5	1	3.5	9	10	0	2	2
2	19	F	no	1.5	0.5	2.	9	3	1	1	5
3	53	F	yes	4.5	4	7.5	14	7	1	7	29
4	51	F	no	4	0.5	4.5	10.	9.5	0	1	8
5	55	F	no	2.5	4	6.5	11	7	2	1	8
6	54	F	no	5	2	7	10	1.5	2	2	9
7	47	F	no	1	5.5	6.5	ND	11.5	ND	2	23
8	20	F	no	1.5	2	3.5	9	2	ND	ND	ND
9	19	F	yes	5	4.5	9.5	13	8.5	ND	1	12
10	48	F	yes	2	1.5	3.5	ND	9.5	0	1	5
11	25	F	yes	4	3	6.5	ND	10.5	0	4	10
12	24	M	no	3.5	2.5	6.	7	2.5	0	3	13
13	34	F	no	2	3	5	10	16.5	ND	0	0
14	27	F	yes	2.5	3.5	6	ND	3.5	2.5	1	1
15	39	F	yes	4	2.5	6	ND	11	ND	ND	ND
16	29	F	yes	5.5	3	8	10	9.5	8	1	4
17	34	F	yes	5.5	1.5	7	11	15	1	2	3
18	37	F	yes	4.5	4.5	8.5	9	4.5	1	1	8
19	53	F	no	2	0	2	10	6.5	0	2	2
20	48	F	no	1.5	3	4	8	4	ND	ND	ND
21	50	F	yes	2	0	2	12	7	ND	ND	ND
22	23	F	yes	1.5	3	4	11	8	ND	ND	ND
23	58	F	yes	4	3	7	9	4	ND	ND	ND
24	32	F	no	2	1.5	3.5	9	4.5	ND	ND	ND
25	21	F	yes	2	0.5	2	11	10	ND	ND	ND
26	52	F	yes	2	0.5	2.5	ND	5.5	ND	ND	ND
27	50	F	yes	6	1	7	9	13	ND	ND	ND
28	54	F	no	3.5	1	4.5	9	9.5	ND	ND	ND
29	43	F	no	5.5	2.5	8	12	2.5	ND	ND	ND
30	37	F	yes	3	2	5	10	2.5	ND	5	13
31	36	M	yes	1.5	2	3.5	9	2.5	ND	1	0
32	38	M	no	2	3	4.5	7	8	ND	0	0
33	44	M	no	1.5	0.5	2	9	6.5	ND	0	1
34	25	F	no	0.5	2	2.5	8	4.5	ND	0	6
35	53	M	no	1.5	2	3.5	8	2	ND	0	2
36	24	M	yes	4.5	2	6.5	8	2	ND	1	1
37	31	M	yes	3.5	0.5	4	9	3	ND	0	2
38	36	M	no	4	2.5	6	8	2	ND	1	3
39	50	M	yes	3	1.5	4.5	ND	6	ND	2	4
40	55	M	no	2.5	3.5	6	25	7.5	ND	ND	ND
41	51	M	yes	1.5	4.5	6	ND	11	ND	ND	ND
42	49	M	yes	0.5	1	1.5	8	9.5	ND	ND	ND
43	42	M	no	1	2.5	3.5	10	6.5	ND	ND	ND
44	37	M	no	2	1	3	ND	5.5	ND	ND	ND
45	25	M	yes	1	1.5	2.5	10	3.5	ND	ND	ND
46	26	M	yes	2.5	2	4.5	ND	6.5	ND	ND	ND
47	26	M	no	0.5	0.5	1	8	5	ND	ND	ND
48	23	M	yes	1	3	4	8	8	ND	ND	ND
49	20	M	no	1	0	1	11	2.5	ND	ND	ND
50	44	M	no	0.5	0.5	1	8	5	ND	ND	ND
51	41	M	no	1	0.5	1.5	10	2	ND	ND	ND
52	39	M	yes	0.5	1.5	2	9	9.5	ND	ND	ND
53	32	M	yes	1.5	0.5	2	8	14	ND	ND	ND
54	24	M	no	1.5	1	2.5	8	3.5	ND	ND	ND
55	23	M	yes	1.5	0.5	1.5	10	14.5	ND	ND	ND
56	21	M	yes	0.5	1	1.5	8	11.5	ND	ND	ND
57	21	M	no	1.5	1	2.5	8	8	ND	ND	ND
58	20	M	no	3	1.5	4.5	8	11	ND	ND	ND

* Individuals Nos. 1-29 are from factory I and Nos. 40-58 are from factory II. Individuals Nos. 1-18 and 40-49 were exposed to ethylene oxide; Nos. 19-39 and 50-58 were controls, see Materials and methods

menopausal complaints, 4 took other drugs without any known mutagenic activity. Nine were smokers.

Controls I A. — These controls were 11 women aged 21–58 (mean 44) years, who worked in another department of the same factory, without exposure to ethylene oxide. Four took hormones, 4 took other drugs. One person had a herpes zoster infection half a year before the examination. Three persons had had X-ray examinations. One person had until 1.5 year before the investigation been employed in a shop selling various chemicals. Six were smokers. A bone marrow sample was obtained from 1 of these controls.

Controls I B. — When comparing the bone marrow parameters a group of 10 bus drivers (8 males and 2 females), described in detail elsewhere (HÖGSTEDT et al. 1981), were employed.

3.2 Factory II

Working conditions. — In this plant the medical equipment was treated with a mixture (11:89 w/w) of ethylene oxide and Freon®12 in three sterilizers. These were unloaded twice a day during approximately half an hour. After sterilization the equipment was taken by truck to a ventilated stockroom, where it was stored for 10 days before packing in another room. The sterilizing personnel had never worn breathing protective devices. Eight of 10 persons worked partly with truck driving (1–2 h/day) and partly with packing. During truck driving they were exposed to ethylene oxide in the stockroom.

Exposed persons. — These consisted of 10 males aged 20 to 55 (mean 36) years, who had been exposed for 0.5 to 8 (mean 1.7) years. Seven of them (all working with truck driving and packing) had been employed just a little more than half a year before the investigation, 2 for about a year and 1 for 8 years. In summary, at biological sampling, 2 subjects were exposed to 8 h TWA levels of 1–2 ppm. One of them had until 2 years before sampling been exposed to considerably higher levels. The others were exposed to levels below 0.5 ppm. One subject had previously been exposed occupationally to inorganic lead until one year before the investigation. He had a blood lead value of 0.17 mg/l. Eight subjects had had X-ray examinations and 1 person took thyroxine because of hypothyreosis. Five were smokers.

Controls II. — Nine male workers from another department of the same factory, who were not exposed to ethylene oxide, were used as controls.

Their age ranged from 20 to 44 (mean 29) years. One of them had possibly had mononucleosis half a year before the investigation, and 1 had had parotitis three months before. Four persons had had X-ray examinations. None of them took any drug. Four were smokers.

4. Cytogenetic methods

4.1 Peripheral blood lymphocytes

Chromosome aberrations. — The same standard microculture technique was used as in previous studies by our group (e.g., HÖGSTEDT et al. 1979). The cultivation time was 72 h. Preparations were coded and analyzed by one observer without any knowledge of the exposure. Two hundred metaphases were scored from each individual and the aberrations were recorded according to the classification system recommended by ISCN (1978). The chromosomal aberrations were referred to either as 'breaks', i.e. chromatid breaks, chromatid interchanges, acentric fragments, ring chromosomes, dicentric chromosomes, and pericentric inversions, or as 'gaps' which included both chromatid and isochromatid gaps. Agreement with another observer was required for scoring a cell as abnormal; observed cells were never rejected. Samples from exposed and control individuals were prepared and analyzed in the same way.

Sister chromatid exchanges (SCE). — The frequency of SCE was studied by the Giemsa-technique essentially according to WOLFF and PERRY (1974). The lymphocytes were stimulated with phytohaemagglutinin and cultivated for 72 h in 5-bromodeoxyuridine at a final concentration of 0.1 mM/ml medium. Twenty metaphases with 46 chromosomes were studied from each individual on coded slides by one observer.

Micronuclei. — The same preparations as for chromosome analysis were used. Two thousand lymphocyte nuclei were identified and the frequency of micronuclei was scored. The micronuclei were of different sizes and before accepting them as micronuclei they were compared with the main nucleus regarding size, structure and color; they had to be not larger than approximately one third of the main nucleus and if they were larger than a small lymphocyte nucleus they had to have almost the same color and structure as the main nucleus. They had to be situated within two nuclear diameters from the main nucleus but clearly separate from it. The preparations were examined with an oil immersion lens (magnification $\times$

1,000). This is essentially the same method as that described by COUNTRYMAN and HEDDLE (1976).

4.2 Bone marrow cells

Chromosome aberrations. — The bone marrow cells used for chromosome studies were obtained from the same sample as the cells for the micronucleus test. The cells, suspended in isotonic phosphate buffer (pH 7) were centrifuged a few hours after collection and incubated overnight in McCoy's medium without any mitogen stimulation. Otherwise, preparations were made in the same manner as for lymphocytes. Twenty-five to 100 mitoses from each individual were analyzed. The aberrations were classified according to the same system as that used for the aberrations in lymphocytes. Gaps were, however, not recorded.

Micronuclei. — Bone marrow cells were obtained by sternal puncture at the same time as the blood samples. The marrow cells were prepared according to the micronucleus test method described by SCHMID (1975) as modified by JENSEN and RAMEL (1976). The cells were suspended in a mixture of equal parts of isotonic phosphate buffer with pH 7 and fetal calf serum. After vigorous shaking, the cells were pelleted and the supernatant removed. The pellet was resuspended in a few drops of buffer and calf serum solution. The cells were smeared on glasses, air-dried, and then the smears were stained with May-Grünwald-Giemsa's stain.

Only interphase cells were analyzed. The micronucleus in a polychromatic erythrocyte was defined as a rounded, bluish or almost black, sharply outlined structure, about $1\mu\text{m}$ across. The micronucleus in an erythroblast was somewhat larger and of the same color as the main nucleus. For each individual 1,000 erythroblasts and 1,000 polychromatic erythrocytes were examined by one observer.

5. Statistical methods

To evaluate the effects of exposure against other types of possible influences on the different parameters expressing cytogenetic damage (chromosomal aberrations, SCE and micronuclei in lymphocytes; chromosomal aberrations and micronuclei in bone marrow cells) analysis of covariance was used (LINDMAN 1974). This analysis included age, smoking, ionizing radiation, drug intake, exposure to ethylene oxide and also the belonging to a certain factory or subgroup of the

material. Because of the unequal proportions of men and women in the different subgroups of the material sex was not possible to include in the analysis of covariance. However, sex is not known to be a predisposing factor to cytogenetic damage (GALLOWAY and EVANS 1975; LATT and JUERGENS 1977; WAKSVIK et al. 1981). Since analysis of covariance demands a normal distribution of the variables the values were transformed into logarithms, which approximately met this requirement. The Spearman rank correlation test was used for the analysis of correlation (SIEGEL 1956, p. 203–213). All tests were two-sided. Statistical significance denotes $P < 0.05$.

Results

Exposed vs. control subjects

Exposed subjects in factory I had higher mean frequencies of cells with breaks, gaps and total number of chromosome aberrations in peripheral blood lymphocytes (Table 2 and 3; Fig. 1 and 2) than controls from the same factory. Also, exposed persons in factory II had higher mean frequencies of cells with breaks, gaps and total number of chromosome aberrations than corresponding controls. Analysis of covariance revealed that the total number of chromosome aberrations and gaps showed significant effect of exposure (Table 3), when other possible background influences (age, smoking habits, etc.) were eliminated. This was, however, not true for breaks.

Neither the frequencies of SCE, nor of micronuclei in lymphocytes showed any significant effect of exposure.

In bone marrow, exposed subjects had significantly higher levels of micronuclei in erythroblasts and in polychromatic erythrocytes (Tables 2 and 3; Fig. 1 and 2).

There was a significant effect of smoking on the frequencies of micronuclei in lymphocytes and in erythroblasts ($P = 0.007$ and 0.04 , respectively). With the other cytogenetic parameters smokers generally had higher values than non-smokers, but the differences were not significant.

Associations between different cytogenetic parameters

Twenty-six correlation coefficients were calculated on the different cytogenetic parameters and are presented in Table 4. Out of 23 positive coeffi-

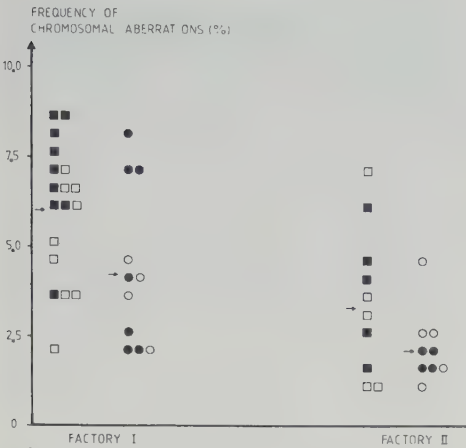


Fig. 1. Chromosomal aberrations in persons exposed to ethylene oxide (squares) and controls (dots). Smokers have closed and non-smokers open symbols. The mean values are indicated by arrows.

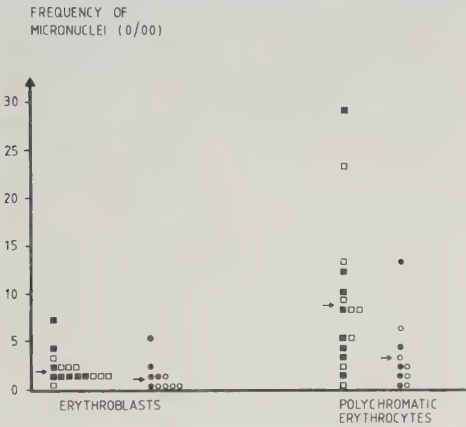


Fig. 2. Micronuclei in erythropoietic bone marrow cells in persons exposed to ethylene oxide (squares) and controls (dots). Smokers have closed and non-smokers open symbols. The mean values are indicated by arrows.

cients 6 were statistically significant with two-sided P values less than 0.05. Three coefficients were negative but not significant. Thus, positive and significant correlations existed between: chromosome aberrations in lymphocytes and bone marrow cells; gaps and breaks in lymphocytes; breaks and SCE in lymphocytes; chromosome

Table 3. Summary of background data and results of cytogenetic tests as well as statistical testing by covariance in persons exposed to ethylene oxide and controls

Peripheral blood lymphocytes						Bone marrow cells with							
No. of individuals	Mean age	No. of smokers	No. of persons with drug intake	No. of X-ray exposed persons	Cells with chromosomal aberrations			SCE	Micro-nuclei (%)	Micronuclei in			
					Breaks (%)	Gaps (%)	Total (%)			Chromosomal aberrations (%)	Erythro-blasts (%)	Polychromatic erythrocytes (%)	
													No./cell
Factory I													
exposed	18	37	9	8	5	3.4	2.7	6.0	10.2	1.4	1.9	8.8	
controls	11	44	6	7	6	2.9	1.5	4.2	10.0	—	—	—	
controls	10	37	5	2	4	—	—	—	—	—	1.0	3.2	
Factory II													
exposed	10	36	5	1	7	1.4	2.0	3.3	11.4	—	—	—	
controls	9	29	4	1	4	1.3	0.9	2.1	8.5	—	—	—	
Two-sided P-values (exposed/controls)						>0.05	0.004	0.008	> 0.05	—	0.050	0.030	

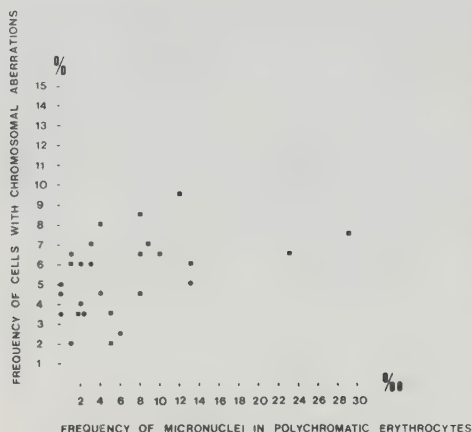


Fig. 3. Association between the frequencies of total chromosomal aberrations in peripheral blood lymphocytes and micronuclei in polychromatic erythrocytes of the bone marrow ($r = 0.47$, $P = 0.014$, $n = 27$) in persons exposed to ethylene oxide (squares) and controls (dots). Smoking habits are not indicated.

aberrations in lymphocytes and micronuclei in polychromatic erythrocytes (Fig. 3); breaks in lymphocytes and micronuclei in erythroblasts; and micronuclei in polychromatic erythrocytes and erythroblasts.

Discussion

This study has shown that, compared to controls, persons exposed to low levels of ethylene oxide have significantly higher frequencies of chromosomal aberrations in peripheral blood lymphocytes and also of micronuclei in erythropoietic bone marrow cells. Our cytogenetic findings emphasize the potential carcinogenicity of ethylene oxide indicated by other studies (HOGSTEDT et al. 1979a, b; DUNKELBERG 1979).

The differences in cytogenetic damage between exposed individuals and controls regarding chromosomal aberrations in lymphocytes was not large. But it was possible to ascertain statistically significant effects as control groups were carefully

Table 4. Rank correlation coefficients* among different cytogenetic parameters in persons exposed to ethylene oxide and controls

Parameter		1	2	3	4	5	6	7	8
<i>Lymphocytes</i>									
Total aberrations	1	—							
Breaks	2	—	—						
Gaps	3	—		0.30 58 0.020	—				
SCE	4	0.25 48 0.091	0.34 48 0.018	0.08 48 0.48	—				
Micronuclei	5	0.06 58 0.63	0.04 58 0.76	0.02 58 0.86	0.07 47 0.62	—			
<i>Bone marrow</i>									
Breaks	6	0.56 14 0.038	0.39 14 0.17	0.53 14 0.054	0.42 11 0.20	— 0.44 13 0.13	—		
Micronuclei in erythroblasts	7	0.36 27 0.064	0.39 27 0.046	0.10 27 0.64	0.41 22 0.060	0.15 26 0.46	— 0.37 14 0.19	—	
Micronuclei in polychromatic erythrocytes	8	0.47 27 0.014	0.31 27 0.12	0.35 27 0.076	0.40 22 0.064	0.13 26 0.53	— 0.14 14 0.62	0.62 27 0.002	—

* The rank correlation coefficient, the number of individuals and the two-sided P-value are given for each possible coefficient

chosen and influences from suspected cytogenetically active factors were eliminated in the analysis.

An interesting observation is the difference in the levels of chromosome aberrations in lymphocytes between the two factories. This difference was present both in controls and exposed persons and had to be considered in the statistical analysis. The difference may be caused by factors related to the culturing of lymphocytes, to socio-economic differences, or by other unidentified factors.

The exposure to ethylene oxide in the two factories was low. According to the admittedly limited data on hand, all 28 exposed persons had been exposed to TWA-levels not exceeding 1 ppm during the last 2.5 years. Thirteen of them had until 2.5 years before the investigation been exposed to higher levels, but probably not exceeding 5 ppm.

The present results showed low but statistically significant coefficients of correlation between several of the parameters studied. There are 26 possible correlation coefficients and 23 were positive. Six of these were statistically significant, which should be compared with the expected frequency of approximately one significant coefficient. This finding shows that there is a non-random association between the cytogenetic parameters. However, the associations are not close. As discussed elsewhere (HÖGSTEDT et al. 1981) this may have several reasons such as: low precision of the determinations and/or different effect and time sequence of the mutagenic agent on the different parameters studied.

The study of bone marrow cells has advantages: The cells are exposed in all phases of the cell cycle; the cells have a rapid turnover and thus preferentially show effects of recent exposure. In addition the scoring of micronuclei is fast and easy and controls do not need to be sampled simultaneously. However, neither the study of chromosome aberrations nor that of micronuclei will become a routine method, since bone marrow samples are difficult to obtain.

Our results indicate that the analysis of chromosomal aberrations in lymphocytes is a sensitive method. The method is, however, time-consuming. The study of SCE is perhaps more rapid, but we found no significant difference between exposed and control subjects by this parameter. This is in accordance with other comparative studies of chromosome aberrations and SCE after chronic in vivo exposure (BRÖGGER 1982).

Micronuclei in lymphocytes may be scored in the same preparations as those used for chromo-

some aberrations. This analysis is rather simple and very rapid. The method has been employed in some studies, which have shown dose related effects of ionizing radiation and chemical mutagens in vitro (COUNTRYMAN and HEDDLE 1976; HEDDLE et al. 1978; LINNAINMAA et al. 1978) and also increased levels in humans after X-ray examinations with iodine-containing contrast medium in vivo (NORMAN et al. 1978). It is interesting to note that in this parameter there was a statistically significant difference between smokers and non-smokers, but no effect of exposure to ethylene oxide nor any inter-correlation with other cytogenetic parameters. As the preparations were intended for chromosome analysis, the culture time was 72 h, while it has been shown that 96 h are necessary to give a maximum of micronuclei (COUNTRYMAN and HEDDLE 1976). Also, since the cytoplasm of the cells has been torn away by the hypotonic treatment, it is difficult to differentiate between real micronuclei and nuclear/cellular debris from other cells. Some micronuclei may also leave the neighbourhood of the main nucleus when the cytoplasm is destroyed. We find the possibility of studying micronuclei in in vivo exposed lymphocytes extremely interesting. Therefore, in future studies a modified technique that leaves the cells intact will be used.

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INCREASED FREQUENCY OF LYMPHOCYTE MICRONUCLEI IN WORKERS PRODUCING REINFORCED POLYESTER RESIN WITH LOW EXPOSURE TO STYRENE

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ABSTRACT

A new micronucleus method based on the analysis of lymphocytes with preserved cytoplasm, revealed an increased frequency of micronuclei in 38 workers employed in a plant producing styrene modified polyester resin as compared to 20 referents (5.9 vs 3.6 o/oo). The time weighted average of the styrene concentration in the workroom air varied between 1 and 36 ppm (mean 13 ppm) during the last year and correlated well to low urinary levels of mandelic acid, which ranged from 9 to 316 mg/g of creatinine (mean 65 mg/g of creatinine).

INTRODUCTION

After the mutagenic and carcinogenic properties of vinyl chloride were revealed, interest was focused on other related chemical compounds such as styrene. By now it is known that styrene and its metabolite styrene-7,8,-oxide have mutagenic and/or carcinogenic effects in different test systems. For a review see the report of Norppa (9). The most important source of occupational exposure to styrene is the production of styrene modified fiberglass reinforced unsaturated polyester resin.

Earlier studies on workers occupationally exposed to styrene have shown increased frequencies of chromosome damage at exposure levels of 25-300 ppm as the 8 h time weighted averages (1, 3, 4, 7). In Sweden the exposure levels have decreased considerably during the last few years.

Micronuclei are thought to be indicators of either chromosome breaking events or failure of the spindle apparatus. In investigations of Countryman and Heddle (2), Linnainmaa et al. (6) and Norman et al. (8) micronuclei in cultured lymphocytes have been analyzed in hypotonically treated cells. Since this treatment destroys the cytoplasm, the method seems to have certain cytological disadvantages.

The aim of the present study was to investigate the effects of styrene at low levels of exposure by a new method for analyzing micronuclei in lymphocytes; the cell cytoplasm was preserved in order to increase the precision of determination. The methodological aspects of the new method will be discussed in a future publication (Högstedt; to be published).

MATERIAL AND METHODS

Subjects studied

The styrene-exposed group consisted of 38 men working in a factory producing fiberglass reinforced polyester resin (table 1). The referents were 20 male workers from a mechanical industry in the same town.

Unsaturated polyester was manufactured by propylene and/or ethylene glycol with maleic and/or phthalic anhydride. Hydroquinone was used as an inhibitor. This alkyd resin was mixed with styrene (70/30 weight/weight). In the factory it was cured by different peroxides (cyclohexanone, or methyl ethyl ketone peroxide, and/or benzoyl peroxide). The curing process was accelerated by heating cobalt naphthenate or dimethyl aniline. The final styrene modified polyester could be treated by drilling or grinding. It could also be glued by epoxy or polyurethane resins. Some of the products were degreased with methylene chloride and sprayed with paints containing different types of organic solvents. Large amounts of acetone were used for cleaning tools. Pressing tools, which were heated to 120° C, were prepared with release agents contaminated with minute amounts of bensene.

Each subject was interviewed regarding occupational and medical history, especially concerning viral infections, use of alcohol and drugs, smoking habits, and exposure to ionizing radiation and heavy metals. Seventeen of the exposed subjects and 8 of the referents were smokers (table 1).

Of the exposed subjects, 11 had had radiographic examinations during the last year and 4 regularly took drugs (ibuprofen, hydrochlortiazid

and atenolol, amitryptilin, and terbutalin). Five of the referents had been examined radiographically and three had a regular drug intake (bendroflumethiazid, metoprolol, and anti-allergic drugs).

One blood specimen and two urine samples (morning and afternoon) were obtained from each subject on either of two days (Tuesday or Wednesday).

Measurements of styrene in workroom air were made in close connection with the biological sampling.

Micronuclei in lymphocytes with preserved cytoplasm

Five drops of whole blood were added to 5 ml of McCoy's 5A medium with 20% newborn calf serum (Flow laboratories). Two cultures were set up for each person. After incubation with 0.135 ml phytohaemagglutinin (Gibco) at 37° C for 72 and 96 h the cells were centrifuged, the medium was removed, and the cells were resuspended in an equal volume of medium, smeared on slides, air dried, and stained in May-Grünwald-Giemsa stain.

The slides were coded and examined by one person with an oil immersion lens with a total magnification of 1,000 x.

The lymphocyte cytoplasm stained blue, and these cells could easily be differentiated from other nucleated cells with segmentation or fragmentation of the nucleus. The micronuclei were of different sizes but had to be smaller than one third of the main nucleus and had to be clearly separated from it. They were of almost the same structure and color as the main nucleus.

Micronuclei in lymphocytes treated with hypotonic saline

The cells were cultured for 96 h by the same microculture technique as already described. Colchicin (0.1 $\mu\text{g/ml}$) was added 1 h before harvest since the preparations also were used for analyzing structural and numerical chromosome aberrations (Högstedt; to be published). Hypotonic treatment was carried out with 0.075 mol/l potassium chloride for 10-15 min at room temperature, and the cells were fixed four times in methanol:acetic acid (3:1). The cells were spread on slides, air dried, and stained in 2 % Giemsa (Merck). The frequency of micronuclei was estimated from the scoring of 2,000 lymphocyte nuclei. Almost the same criteria as already given were used for the micronuclei. However, since the cytoplasm was lacking, the micronuclei had to be situated within two nuclear diameters from the main nucleus. The preparations were coded and examined with the same magnification as was used for the cells with preserved cytoplasm and by the same observer.

Air styrene determinations

The workroom air concentrations of styrene were determined for all types of work in close connection with the blood sampling in 1980. Altogether, 262 air samples were collected by stationary and personal sampling with a total sampling time of 307 h. The samples were analyzed either by an infrared spectrophotometric or a gas chromatographic method. The detection limit was below 1 ppm. Measurements had been done previously with combustible gas indicator and reagents tubes (in 1970) and gaschromatographic methods (in 1975, 1976, 1977, and 1979). The levels found for the individuals in the study on

different sampling occasions are shown in table 2. The measurements in 1970-1979 were performed during short times and only in spots where high exposure were expected. Only the results from 1980 are time weighted averages for 8 h of exposure, and refer directly to the individuals in the present study.

Mandelic acid in urine

Urine samples were obtained in the afternoon after 6 h of work (Tuesday and Wednesday) and on the following morning immediately after the subject awakened. Mandelic acid was determined by a high pressure liquid chromatography method (10). The limit of detection was 5 mg/l. The accuracy was checked by interlaboratory comparison. The levels of MA were related to urinary creatinine levels (13).

Relationship between styrene in air and mandelic acid in the urine

There was a statistically significant correlation between the measurements of mandelic acid in the afternoon and morning urine ($r = 0.73$, $N = 37$, $P = 0.002$) and between these measurements and the levels of styrene in the workroom air at the time of the investigation ($r = 0.64$, $N = 37$, $p = 0.002$, and $r = 0.41$, $N = 37$, $p = 0.012$, respectively).

Individual styrene exposure

For each worker approximate concentrations of styrene in the workroom air for each worker during the period 1970-1980 were estimated on basis of occupational history and styrene levels in the plant. Thus we calculated the mean concentrations of the last 1, 3 and 10 years as well

as the accumulated doses (parts per million x years) during the last 3 and 10 years. During the last 5 years no one in this study had an exposure level exceeding a time-weighted average of 40 ppm. The mean time-weighted average level in the last year (1980) was 13 ppm (range 1-36 ppm)-(table 2).

Statistical analysis

To evaluate the effects of styrene exposure, smoking, and age, on the cytogenetic variables, a multivariate analysis was performed (5). Since this type of analysis demands normal distribution of the variables, the values were transformed into logarithms to approximately meet this requirement.

The styrene exposed group of individuals was divided into two groups. The first had been working not more than 5 years (N= 16) and thus not been exposed to styrene levels exceeding a time-weighted average of 40 ppm. The second consisted of persons who had been exposed for more than 5 years (N= 22).

The effect of exposure was also tested using the different individual dose estimates mentioned in the section on individual styrene exposure, the values of mandelic acid in the morning and the afternoon urine samples and the time of employment.

In the analysis of correlation the Spearman rank correlation coefficient was used (12).

Statistical significance level used was $P < 0.05$. All tests were two-sided.

RESULTS

There was a significant effect of styrene exposure on micronuclei in lymphocytes with preserved cytoplasm cultured for 96 h ($P = 0.005$) (table 1, fig 1) when other potentially influencing factors such as smoking and age were eliminated in the multivariate analysis. Even in individuals who had been working for only 5 years or less ($N = 16$) and thus had never been exposed to styrene levels exceeding a time-weighted average of 40 ppm, there was a difference between the exposed and the reference group (means 6.3 ± 3.6 o/oo; $P = 0.028$).

In lymphocytes with preserved cytoplasm cultured for 72 h and in hypotonically treated cells the increase of micronuclei was not statistically significant (table 1, fig 1).

In the study of dose-response relationships between styrene exposure and micronuclei, the effects of the different individual dose estimates were analyzed separately for the exposure group. No such relationships were found between the different dose estimates, ie. exposure time, individual styrene air levels (during the last one, three, and ten years), the individual accumulated doses (parts per million x years in the last 3 and 10 years), or urinary mandelic acid levels (in morning or afternoon) on one hand, and the frequencies of micronuclei on the other.

DISCUSSION

The main result of this investigation was the increase of micronuclei in lymphocytes with preserved cytoplasm after low exposure to styrene. Smoking and age, which were allowed for in the statistical analysis also had significant effects on the frequencies of micronuclei (Högstedt; to be published). We have earlier shown an increased level of carcinogen induced unscheduled deoxyribonucleic acid synthesis in blood specimens collected at the same time from the same group of individuals (11).

The present exposure level (mean 13 ppm during the last year) associated with cytogenetic effect is lower than in any published study of structural chromosome aberrations among styrene exposed workers. The accuracy of the estimates of recent exposure is supported by low and correlated levels of styrene in the air and mandelic acid in the urine. The estimates of earlier exposure are probably not as accurate. However, the information is sufficient to conclude that the mean exposure level did not exceed 40 ppm for any individual during the last 5 years. In addition, those individuals who had been working in the factory for less than 5 years showed a statistically significant effect for styrene.

No dose-response relationship was found in this study between different individual dose estimates of styrene and micronuclei in lymphocytes. This lack of relationship was not surprising, as the dose range was rather narrow. In addition, there was a lack of precision in the individual dose estimates of the earlier years, and in the measurements of micronuclei as well. Furthermore, the effect might have been caused by a confounding factor in the work environment. Thus there were several chemical compounds with potential mutagenic activity (see the "Material

and Methods" section). It must be stressed however that styrene by its high vapor pressure was the main pollutant in the workroom air.

The hygienic standard for styrene in Sweden has recently been reduced to 25 ppm. Our results suggest that, from an international point of view, not even this very low level will supply full protection against cytogenetic damage.

The present results are consistent with another study on the effect of low styrene levels (Nordenson; personal communication); it revealed an increase of micro-nuclei in lymphocytes with preserved cytoplasm and an increase of cells with aneuploidy but not an increase of structural chromosome aberrations. Our results together with those of Nordenson, may suggest that exposure to styrene at a low level preferably causes disturbances of the spindle apparatus, but not necessarily chromosome breakage.

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Table 1. Summary of data on the styrene exposed subjects and the referents.

	<u>Exposed group</u> (N=38)		<u>Reference group</u> (N=20)	
	Mean	Range	Mean	Range
Age (years)	38.7	19-63	36.4	22-59
Smokers	17		8	
Exposure time (years)	7.9	1-23	-	
Time-weighted average of the styrene levels in air in 1980 (ppm)	13	1-36	-	
Urinary mandelic acid levels in 1980 (mg/g creatinine)	65	9-316	-	
Micronuclei (o/oo)				
In hypotonically treated cells	4.3	0.5-18	3.7	0-8
In cells with pre- served cytoplasm				
72 h	3.9	0-12)	3.0	0-14
96 h	5.9	1-13	3.6	0-8

Table 2. Styrene levels in the workroom air on different sampling occasions. Only the values from 1980 are 8 h time-weighted averages. Regarding dose estimations see the section on individual styrene exposure.

Type of work	Styrene level (ppm)											
	1970		1975		1976		1977		1979		1980	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Hand Lay-up												
1.	130	30-320	44	7-93	27	2-49	21	17-24	41	8-71	16	15-16
2.	-	-	-	-	34	24-60	-	-	72	59-78	13	7-19
3	-	-	-	-	-	-	-	-	-	-	6	3-8
Spray-up	-	-	33	12-79	-	-	-	-	-	-	9	7-13
Pressing	-	-	-	-	-	-	-	-	-	-	15	4-36
Cold pressing	-	-	17	14-20	-	-	31	1-78	-	-	5	5-6
Hot pressing of sheet molding compounds	-	-	-	-	14	2-24	-	-	-	-	11	4-15
Total sampling time (h)	12		64		44		31		66		307	

FREQUENCY OF
MICRONUCLEI (‰)

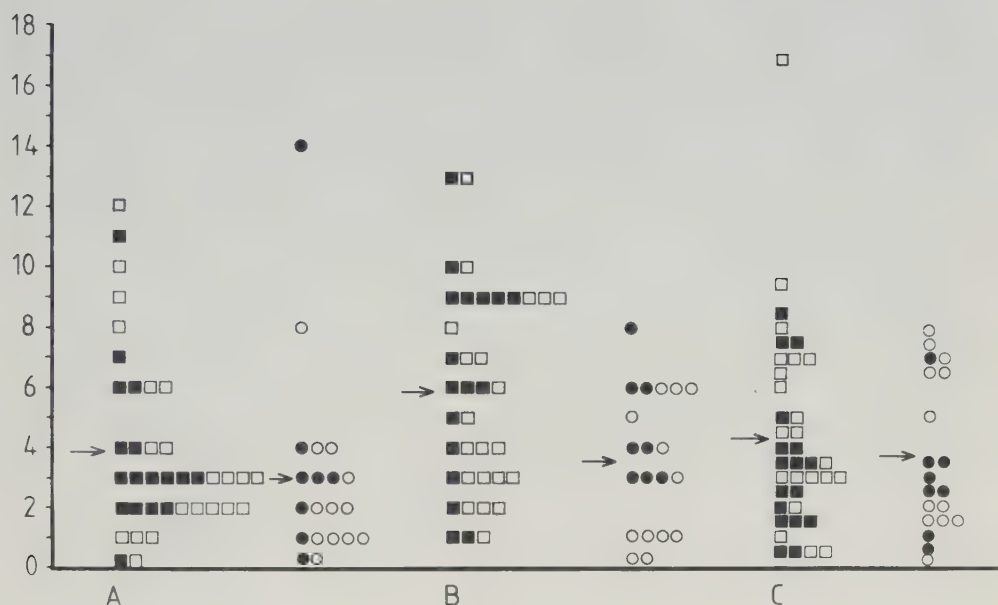


Fig 1. Frequency of micronuclei. (A = micronuclei in lymphocytes with preserved cytoplasm cultured for 72 h, B = micronuclei in lymphocytes with preserved cytoplasm cultured for 96 h, C = micronuclei in hypotonically treated lymphocytes cultured for 96 h, closed squares = styrene exposed smokers, open squares = styrene exposed nonsmokers, closed circles = nonexposed smokers, open circles = nonexposed nonsmokers)

MICRONUCLEI IN LYMPHOCYTES WITH PRESERVED CYTOPLASM - A METHOD FOR ASSESSMENT OF CYTOGENETIC DAMAGE IN MAN

by
Benkt Högstedt

SUMMARY

The micronucleus method for studying cytogenetic effects in lymphocytes in man was modified. The cells were analyzed with preserved cytoplasm, which allowed a more precise identification of micronuclei. The method was tested on 58 individuals 38 of whom were exposed to styrene. Higher frequencies of micronuclei were obtained in 96 h cultures than in 72 h ones. In cells X-rayed in_vitro a culture time of 80 - 88 h gave a maximal frequency of micronuclei. There was a very close correlation between the results of cultures from whole blood and from "buffy coat" ($r = 0.99$). There was also a good correlation between two observers ($r = 0.95$) but a systematic difference of about 30% existed between the two sets of observations. The method error (variation coefficient) was 11 and 6 % in preparations with mean micronuclei frequencies of 4 and 38 o/oo, respectively. The styrene exposed group displayed weak but statistically significant correlations between frequencies of micronuclei and numeric chromosome aberrations. There were statistically significant effects of age, smoking and low levels of styrene exposure but these factors explained only 12-24 % of the total variance.

INTRODUCTION

There is a great need for simple and informative methods for studies in man of mutagenic events in somatic and germinal cells. Chromosome aberrations and sister chromatid exchanges in cultured lymphocytes are generally used methods when studying mutagen effects in humans exposed in vivo. Another possibility is to use micronuclei as an indirect indicator of chromosome damage. Micronuclei are formed during cell division from acentric fragments or lagging chromosomes. They are easily detected in interphase cells as free intracytoplasmatic bodies.

Micronuclei in erythropoietic bone marrow cells are good indicators of cytogenetic damage in man (Högstedt et al., 1981a-c, 1983a) however the difficulty to obtain bone marrow samples makes the method impossible to use in large scale investigations. Since the formation of micronuclei is a general phenomenon in dividing, damaged cells it is also possible to study them in cultured lymphocytes.

Countryman and Heddle (1976) and Heddle et al. (1978 b) have shown that X-ray irradiation and mitomycin C in vitro gives a dose related increase of micronuclei in cultured lymphocytes. They scored micronuclei in cells after the cytoplasm had been destroyed by hypotonic treatment. This method was used by Norman et al. (1978), who found an increased level of micronuclei in patients after X-ray examinations with iodine-containing contrast media. Linnainmaa et al. (1978) found a dose related effect in lymphocytes after in vitro exposure to styrene. We have earlier used this method in an in vivo study which showed an increased frequency of micronuclei in smokers compared to nonsmokers (Högstedt et al., 1983a).

Scoring micronuclei in hypotonically treated preparations, displayed certain disadvantages. It was often difficult to differentiate real micronuclei from nuclear/cellular debris from other cells, since the cytoplasm of the cell was lacking. Cells with segmentation or fragmentation of the nucleus could easily be regarded as lymphocytes with micronuclei. Since the cytoplasm of the cells were destroyed, micronuclei might also be torn away from the neighborhood of the original nucleus.

A new method is hereby presented involving analysis of micronuclei in cultured lymphocytes with preserved cytoplasm. The results of this method are compared to those obtained with the method of hypotonically treated lymphocytes. The questions of cytological difficulties, culture time, method error, interobserver variation, difference between culture techniques, time needed for analysis, relation to other methods expressing mutagenic activity are discussed as well as factors influencing the variation of micronuclei frequencies.

METHODS

A. Micronuclei in lymphocytes with preserved cytoplasm

1. In vivo exposed individuals

Whole blood was added to McCoy's 5A medium with 20% new-born calf serum. Two cultures were prepared for each person. After incubation with PHA in 37 °C for 72 or 96 h the cells were centrifuged, the medium was removed and the cells were suspended in an equal volume of medium, smeared on slides, air dried and then stained with May-Grunwald-Giemsa stain. The slides were

examined with an oil immersion lens with a total magnification of $\times 1,000$.

2. In vitro studies

Venous blood was sampled from two healthy donors. Whole blood cultures as well as cultures with cells from "buffy coat" were prepared and analyzed as described above.

B. Micronuclei in lymphocytes treated with hypotonic saline

The same microculture technique as above was used. The cells were cultured for 96 h. Colchicin (0.1 $\mu\text{g/ml}$) was added 1 h before harvest. Hypotonic treatment was carried out with 0.075 mol/L KCl for 10-15 min in room temperature and the cells were fixed in methanol:acetic acid (3:1). The cells were spread on slides, air dried and stained in Giemsa. The frequency of micronuclei was estimated by scoring 2,000 lymphocyte nuclei. Almost the same criteria as for micronuclei in whole cells were used, but since the cytoplasm was lacking, the micronuclei had to be situated within two nuclear diameters from the main nucleus. The preparations were examined with a magnification of $\times 1,000$. This method was only applied on the in vivo exposed individuals.

In these preparations also structural and numerical chromosome aberrations in 100 metaphases per individual were analyzed according to methods previously described (Högstedt et al., 1979).

C. Statistical methods

To evaluate the effects of smoking, age and occupational exposure on the cytogenetic parameters, a multivariate analysis was performed as described earlier (Högstedt et al., 1983 a).

In the analysis of correlations the Spearman rank correlation coefficient was used.

Statistical significans denotes $P < 0.05$. All tests were two-sided.

MATERIALS

A. In vivo exposed individuals. Samples were obtained from 58 male workers (table 1). 20 of these (group A) were employed in a mechanical industry. Their ages were 22-59 (mean 36.4) a. In addition, 38 styrene exposed workers in a plastic factory were studied (group B). They were 19-63 (mean 38.7) a. Further details on the groups are given elsewhere (Högstedt et al., 1983 b), together with data on age, smoking habits, and styrene exposure. Blood specimens from both groups were obtained in two following days.

B. In vitro material. Blood samples from two healthy subjects were studied after irradiation in vitro with 0-3 Gy.

RESULTS AND COMMENTS

A. Cytological findings

The lymphocytes stained with light blue cytoplasm and red-brown nucleus. It was possible to differentiate between activated and non-activated lymphocytes. Polynuclear leukocytes could easily be distinguished from lymphocytes. Also, other types of cells of unclear origin with segmen-

tation or fragmentation of the nucleus were easy to detect by the May-Grunwald-Giemsa staining and could be separated from lymphocytes with micronuclei. The micronuclei were of different sizes but had to be smaller than one third of the main nucleus. The advantage of the new method was the possibility to score only lymphocytes and to analyze intracytoplasmatic micronuclei safely.

B. Culture time

In the in vivo exposed group there was statistically significant higher frequencies of micronuclei after 96 h than after 72 h ($p = 0.010$; table 1). However in 13 individual cases there were higher frequencies after 72 h. When different culture times were investigated in normal cases and after in vitro irradiation it was obvious that the maximal frequencies of micronuclei are obtained between 80 and 88 h. Fig. 1 a and b shows the frequencies of micronuclei at different culture times after irradiation with 0 and 2 Gy.

Countryman and Heddle (1976) found a linear increase of micronuclei until 96 h. In a later publication, however, a culture time of 84 h was found to give a maximal level of micronuclei (Heddle et al., 1978 a).

The turn over of micronuclei in cultured lymphocytes is thus apparently similar to that of micronuclei in bone marrow cells after genotoxic exposure (Fliedner et al., 1964; Tunek et al., 1982).

C. Whole blood vs. "buffy coat" cultures

Fourteen double cultures were set up and some of them were irradiated with 0-3 Gy. The first set of cultures was whole blood cultures and the se-

cond was set up with cells from the "buffy coat" layer. The 28 cultures were analyzed by one observer. The results are given in fig. 2. The correlation coefficient between the two scores was very high ($r=0.99$) and there was no systematic difference between the two methods.

It is often very easy to get white blood cells (both lymphocytes and polynuclear leukocytes) from the "buffy coat" layer. Especially when the heparinized venous blood is fresh, the cells spontaneously separate within 1 h.

D. Method error

The method error consists of two components, i.e. the error caused by the culture procedure and the error of the analysis. When lymphocytes are cultured in medium and with PHA from identical batches the culture depending part of the total method error is neglectable as can be seen in fig. 2, which shows the results of double cultures.

In order to investigate the analysis depending part of the method error the variation coefficient ($100 \times \text{standard deviation} / \text{mean}$) was calculated on repeated measurements in the same individual.

In two sets of preparations with mean micronuclei frequencies of 4 and 38 o/oo, 10 measurements were made and the variation coefficient were calculated after different number of cells had been analyzed (fig. 3). When 1,000 cells had been analyzed the method error was 26 and 10 %, respectively, and scoring 3,000 cells reduced the variation coefficients to 11 and 6 %, respectively.

It can be mentioned that in the in_vivo exposed material the effect of styrene exposure (see below) was statistically significant already when 500 cells were analyzed ($p= 0.05$). The final significance was reached after 750 cells and did not further increase when 1,000 cells were scored ($p= 0.005$). If the difference between the groups had been smaller (table 1) and/or less individuals had been studied a statistical significance could still have been obtained if larger number of cells had been analyzed.

E. Inter-observer_variation

Preparations of 24 cultures with different degrees of irradiation were scored independently by two observers. The results are given in fig. 4. The correlation coefficient between the scores was high ($r= 0.95$) but there seemed to be a systematic difference, as one of the observers identified about 30% fewer micronuclei than the other. This shows that materials ought to be scored by one observer.

F. The_time_needed_for_analysis

A good preparation of a whole blood culture can be analyzed in less than 1 h (1,000 cells). With "buffy coat" culture preparations the corresponding time needed is around 10 min, since the lymphocytes are much closer together.

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F. The time needed for analysis

A good preparation of a whole blood culture can be analyzed in less than 1 h (1,000 cells). With "buffy coat" culture preparations the corresponding time needed is around 10 min, since the lymphocytes are much closer together.

G. Relation between the different micronuclei methods and other methods expressing mutagenic activity

There were weak but significant correlations between micronuclei in lymphocytes with preserved cytoplasm cultured for 72 h on one hand and micronuclei in cells cultured for 96 h prepared

with ($r = 0.30$, $P = 0.02$) and without cytoplasm ($r = 0.26$, $P = 0.04$) on the other. Also, there were positive correlations between the frequencies of cells with numeric chromosome aberrations after 96 h and micronuclei in hypotonically treated cells as well as in lymphocytes with preserved cytoplasm, cultured for 72 h ($r = 0.30$, $P < 0.05$ and $r = 0.27$, $P < 0.05$, respectively). There was no correlation between any kind of structural chromosome aberrations and micronuclei. Neither was there any correlation between the cytogenetic parameters mentioned and the two different parameters of DNA repair (carcinogen and UV induced unscheduled DNA synthesis; Pero et al., 1982), which were analyzed at the same time on the same group of individuals.

The relative lack of correlation between the parameters may be explained by the low precision of the individual measurements that certainly will interfere with associations. Further, the effect parameters might be differently affected by factors such as chemical exposure, smoking and age. The structural chromosome aberrations were analyzed in cells cultured for 96 h, which may affect the association with the frequencies of micronuclei. However, in an earlier study (Högstedt et al., 1983a) we did not find any correlation between micronuclei and structural chromosome aberrations in cells cultured for 72 h.

The association between micronuclei and the frequencies of cells with numeric chromosome aberrations is interesting. This indicates a relation between lagging chromosomes on the one hand and the formation of either micronuclei or cells with numeric aberrations on the other. An additional explanation might be that cells with abnormal karyotypic pattern are easier damaged and therefore show higher mutagenic activity than normal ones, which is consistent with our earlier findings regarding micronuclei in leukemic patients (Högstedt et al., 1981a,b).

H. Factors influencing the variation of micronuclei

In the group of in vivo exposed individuals the multivariate analysis revealed a statistically significant effect of styrene at a low exposure level ($P = 0.005$) and of smoking ($P = 0.014$) on micronuclei in lymphocytes with preserved cytoplasm cultured for 96 h. In this parameter there was also a possible effect of age, however not significant ($P = 0.11$). Also, in micronuclei in cells with preserved cytoplasm cultured for 72 h and in hypotonically treated cells there was a difference in means between styrene exposed and controls but it was not statistically significant. In these two parameters there was a significant effect of age ($P = 0.048$ and $P = 0.002$, respectively). Neither drug intake, recent X-ray examinations, nor blood lead values (data not presented) had any effect on the micronuclei parameters.

Smoking is an possible source of mutagenic/carcinogenic impact in man. Also, it has proved to have effects in some cytogenetic parameters, particularlyly sister chromatid exchanges (Lambert et al., 1978; Husgafvel-Pursiainen et al., 1980). We have also in earlier studies been able to show the effect of smoking on micronuclei, both in peripheral blood lymphocytes and in erythropoietic bone marrow cells (Högstedt et al., 1983 a). Since, statistically, it is possible to separate the effect of smoking on the cytogenetic variables, smokers could be used as positive controls.

The age effect on micronuclei may be caused by either accumulated mutagenic damage or by true ageing processes such as altered cell meta-

bolism and/or decreased repairing capacity. There may also be combinations between these possibilities. Other cytogenetic studies on chromosome aberrations and sister chromatid exchanges have shown age effects (for review see Hedner et al., 1983), although in most studies it has not been considered.

The effect of styrene exposure is discussed elsewhere (Högstedt et al., 1983 b).

From the multivariate analysis it could be calculated that 12-24% of the variation in micronuclei could be explained by the factors styrene exposure, smoking and age (table 2). The rest of the variation may be caused by a considerable method error and also by unknown influences such as food habits, individual variation in metabolic activation of mutagens and other factors influencing the individual susceptibility.

The large proportion of unexplained variance including the method error certainly reduces the probability to obtain statistical significance in studies of mutagenic agents.

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Table 2.
 Variance (%) of micronuclei frequencies explained by
 styrene exposure, smoking and age and two-sided
 P-values in a material of 58 individuals

	Micronuclei in		
	Lymphocytes with preserv- ed cytoplasm cultured for 72 h N=58	lymphocytes cultured for 96 h N=58	hypotonically treated lymphocytes N=58
Styrene exposure	3 (P= 0.16)	10 (P= 0.005)	1 (P= 0.47)
Smoking	2 (P= 0.34)	10 (P= 0.014)	0 (P= 0.95)
Age	7 (P= 0.048)	4 (P= 0.11)	20 (P= 0.002)
Total	12	24	21

Table 1.

Micronuclei (0/000) in lymphocytes with preserved cytoplasm and in cells treated with hypotonic saline

S.D.= standard deviation. 1,000 cells were analyzed in every individual.

	N	Cells with preserved cytoplasm				Cells treated with hypotonic saline			
		72 h		96 h		96 h			
		Mean	S.D.	Range	S.D.	Mean	S.D.	Range	
Group A	20	3.0	3.1	0-14	2.3	3.6	2.3	0-8	0-8
Group B	38	3.9	2.9	0-12	3.2	5.9	3.2	1-13	0.5-18
Total A + B	58	3.6	3.0	0-14	3.2	5.1	3.2	0-13	0-18

Micronuclei in
5,000 cells

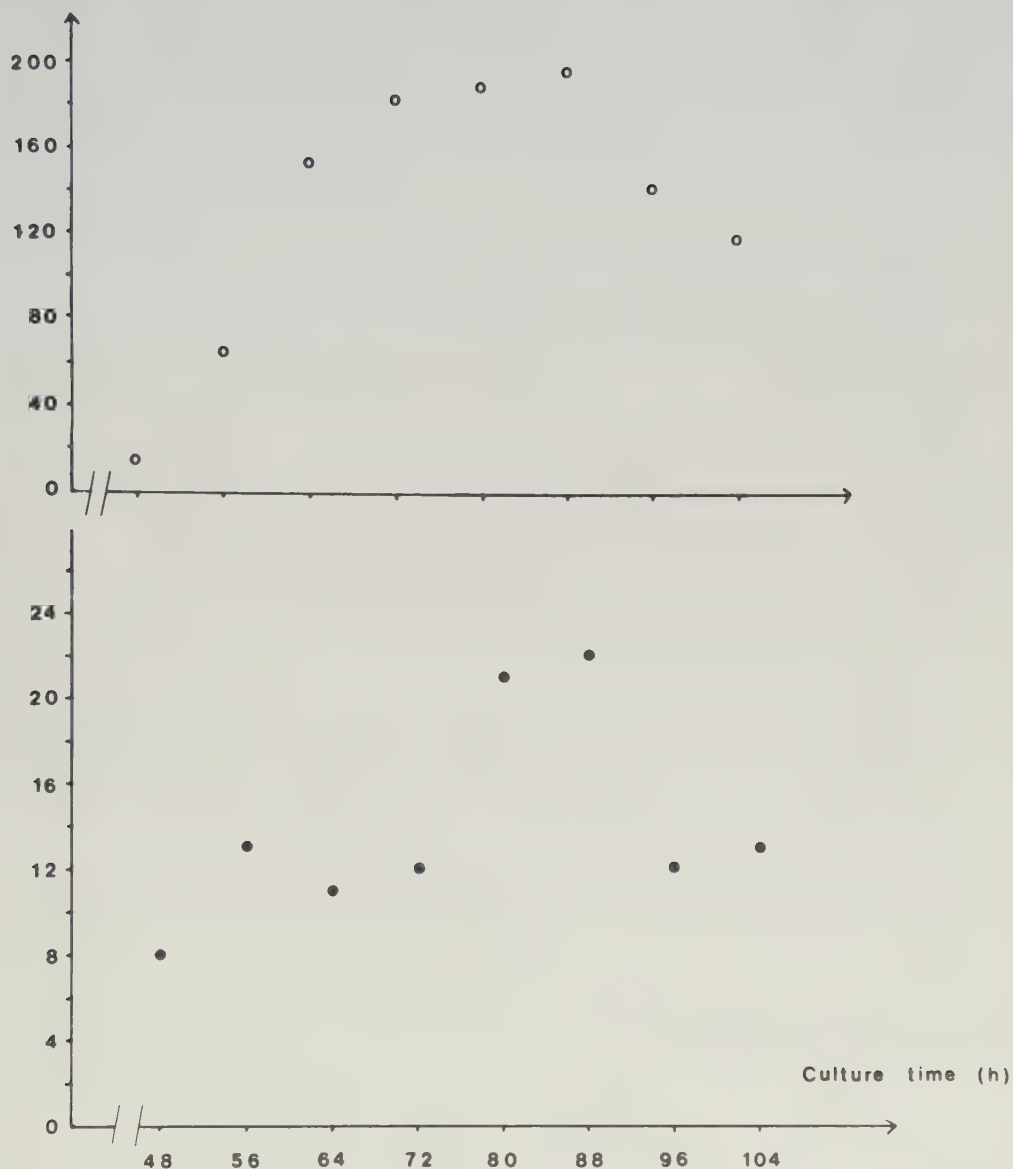


Fig. 1 A, 1 B

Frequencies of micronuclei on different culture times in cultures treated with 2 Gy (1 A) and controls (1 B).

Micronuclei (‰) in
lymphocytes from
whole blood

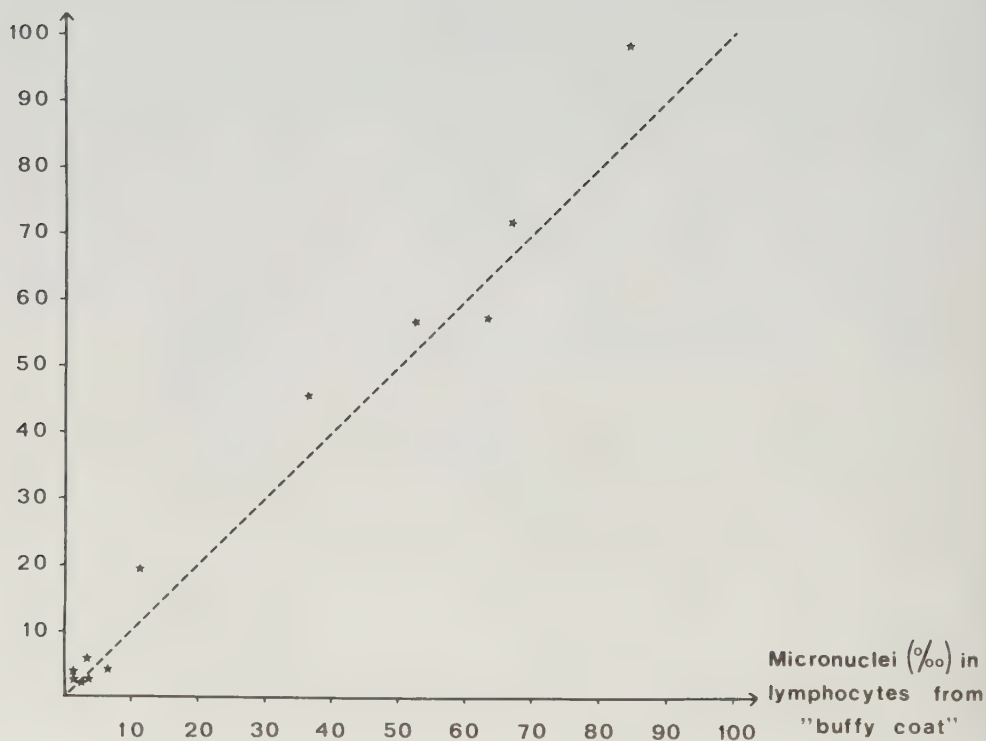


Fig. 2

Results of 14 double cultures set up either with whole blood or cells from the "buffy coat" layer, ($r = 0.99$).

VARIATION
COEFFICIENT

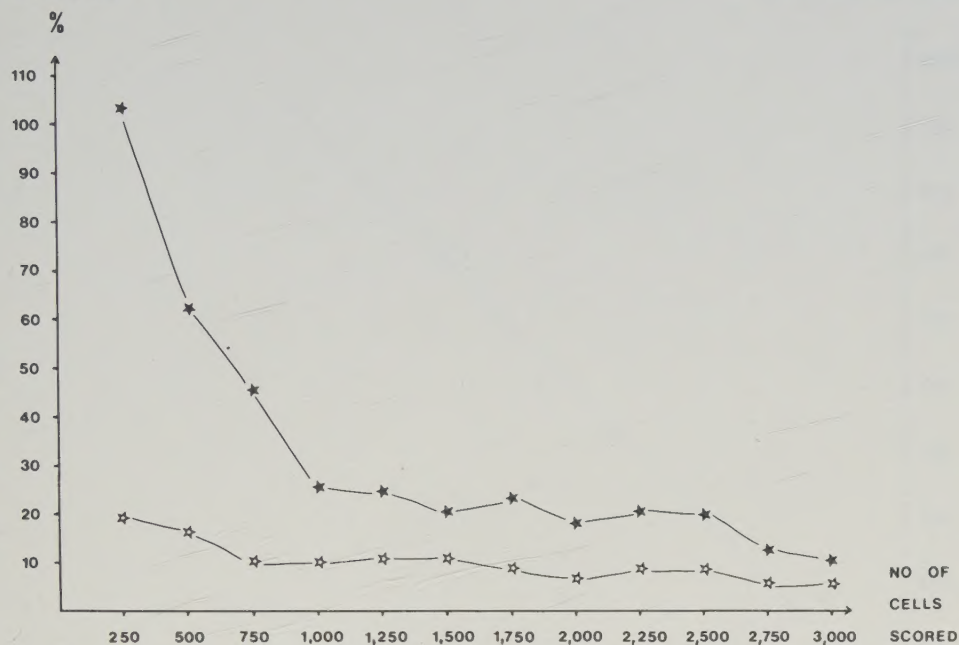


Fig. 3

Variation coefficient ($100 \times S / M$) after scoring different number of lymphocytes in two samples with mean micronuclei frequency of 4 o/oo (upper line) and 38 o/oo (lower line).

2. Observer
Micronuclei (‰)

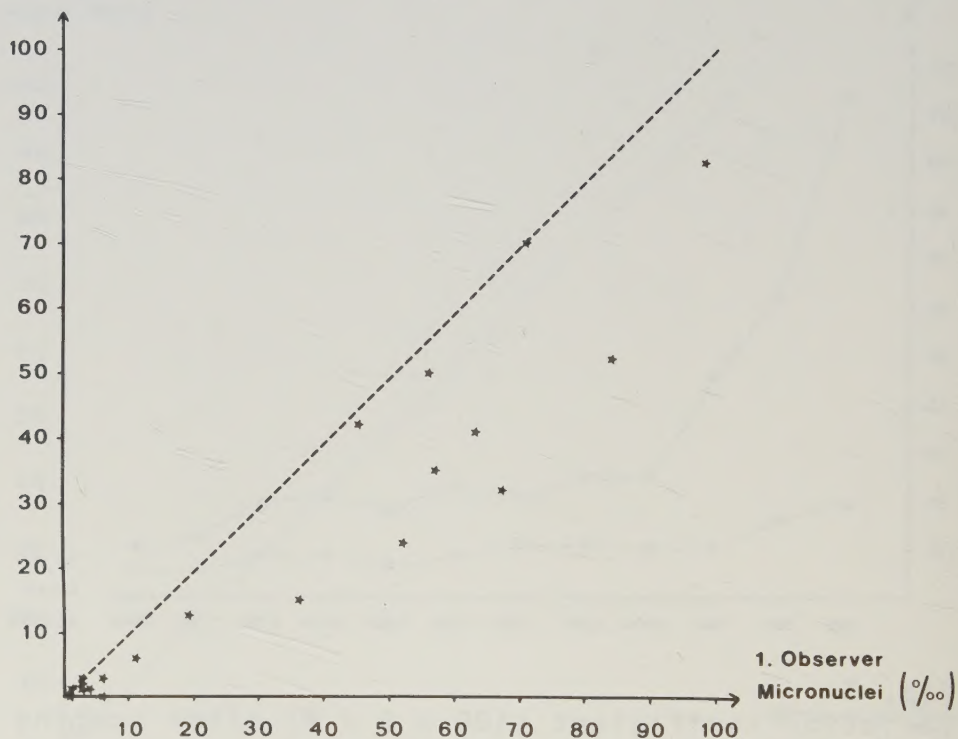


Fig. 4

Analysis of 24 preparations by two independent observers, ($r = 0.95$).

